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THE UNIVERSITY OF ALBERTA

ANTIBODY MEDIATED CELL-DEPENDENT IMMUNE LYMPHOLYSIS IN HUMAN

by

THAVISAKDI KOVITHAVONGS (C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ANTIBODY MEDIATED CELL DEPENDENT IMMUNE LYMPHOLYSIS IN HUMAN submitted by THAVISAKDI KOVITHAVONGS in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences.

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Date: April 16, 1976

To

my wife, Orranoot, for
her understanding and
encouragement; and our
children, Kay and Carl,
for their patience.

ABSTRACT

The phenomenon of antibody induced cell mediated cytotoxicity has been applied to the study of tissue sensitisation in humans, using ^{51}Cr release assay from peripheral blood lymphocyte targets, in a test system called "antibody mediated cell dependent immune lympholysis" (ABCIL). The effector cells active in this test system are lymphocytic, non-adherent and functionally radiosensitive. They are probably identical to the K1 cells recently described.

Compared with the conventional complement dependent lymphocytotoxicity test, this system is much more sensitive and can detect other specificities in addition to HLA-A&B. This type of antibody also appears earlier following tissue sensitisation and persists longer after cessation of antigenic exposure. Through studies with family segregation, differential platelet and lymphocyte absorptions, and correlation with studies in mixed lymphocyte culture tests (MLC) and serum inhibition in one-way MLC, convincing evidence has been obtained, which indicates that this test system may detect HLA-D antigens, or MLC gene products. Evidence also suggests that by testing unknown cells with certain selected sera in tandem before and after serum absorption with HLA-D homozygous cells, it may become possible to type for certain HLA-D antigens on unknown cells.

In hemodialysis and transplanted patients, many of the so-called "non-responders" who did not produced complement dependent cytotoxic antibody are, in fact, "ABCIL responders" who produce ABCIL antibody.

In contrast to findings by others, the detrimental role of this antibody in the field of kidney transplantation cannot be substantiated in this study. It is speculated that there may be two types of ABCIL antibody, those directed against HLA-A&B which may be harmful while others against HLA-D which may be beneficial.

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Chapter I. Introduction and Review of the Literature

A. Introduction

Since the discoveries of the importance of the thymus (118) and the bursa of Fabricius (63), it has been conceptually entertained that the bodily defense mechanism is dichotomized into a humoral antibody system and a cell-mediated immune system (58). To certain types of infection, or immunization, the humoral antibody is the predominant mediator of immunity, and this is produced by the B (Bone-marrow derived or Bursa equivalent) - lymphocyte. The cell-mediated mechanisms are characteristic of delayed hypersensitivity, homograft immunity and graft-versus-host reactions and are carried out by the T (Thymus derived) - lymphocytes. The destruction of cells or micro-organisms in the former instance is accomplished by a special class of protein, collectively termed "the complement system" (61), after interaction has taken place between the antibody and the target. This leads to phagocytosis or cell lysis. In the latter, T-cells (28, 119) are responsible for the direct killing of the target cells in contact by a process as yet not entirely understood, as well as releasing factors or lymphokines (39) to facilitate target destruction by other means.

In this thesis, another mechanism of target cell killing will be introduced which incorporates the humoral antibody and lymphoid cells in mediating cytotoxicity, without the participation of complement (177, 196, 199). Although in most studies, this has

been an *in vitro* phenomenon, there is evidence to suggest that it may be operative in vivo as well. Further, it is the intention of the author to show evidence and ways of how this in vitro system can be used to study tissue sensitization with the emphasis on detection of the HLA antigens of the D locus which controls allogenic stimulation in mixed leukocyte culture (MLC). Evidence is accumulating that compatibility at this D locus may be more important to graft survival in organ transplantation than compatibility at the other loci of the HLA-A, B and C.

B. Antibody-induced cell-mediated cytotoxicity

1) Observation by E. Möller

In 1965, Erna Möller (122) at the Karolinska Institute reported on the cytotoxicity by normal lymph node cells against tumor cells in culture, when the tumor cells and normal lymph node cells were brought in contact by antibody. In her tissue culture system, sarcoma cells from one mouse strain were killed by immune lymph node cells from another strain. In addition, these sarcoma cells were also killed by normal allogeneic lymphoid cells in the presence of heat inactivated heterologous rabbit antiserum raised against mouse lymphoid cells. The heterologous antiserum alone without the normal allogeneic lymphoid cells, and vice versa, did not have any cytotoxic effect. Möller interpreted this as representing cytotoxicity brought about by contact of lymphoid cells against histoincompatible targets, since in her experiments, isologous lymphoid cells did not produce any cytotoxicity against the sarcoma cells in the presence of rabbit antiserum.

Although Möller, herself, failed to indicate as such, the observation was the first to show the interaction between humoral antibody and lymphocytes in causing target cell destruction, without the participation of serum complement components. This was confirmed, three years later, by work done separately by Perlmann (135) and MacLennan (107), using radioactive chromium (^{51}Cr) release assay to be described below.

2. Confirmations by Perlmann and MacLennan

Although Govaerts (71) in 1960 had already observed cytotoxicity by normal lymphoid cells in the presence of immune serum, his findings did not allow such conclusion to be made, since serum complement was also present in the cultures. Möller's observation was confirmed by Perlmann and MacLennan who also did extensive study in this cytotoxic system.

Perlmann (135) immunized guinea pigs repeatedly with BCG or PPD. Immune spleen cells or blood lymphocytes from such animals were cytotoxic to fowl erythrocytes coated with PPD as judged by the increased ^{51}Cr release over controls (with normal guinea pig spleen cells). Uncoated target cells were not affected indicating that cytotoxicity was directed against the PPD on the red cell surface. These immunized animals also produced antibody against PPD in the hemagglutination test and the hemolytic test in the presence of complement. When these antisera were heat inactivated then added to cultures with antigen coated target cells and normal guinea pig spleen cells, cytotoxicity was again detected and could be blocked by adding PPD or rabbit antiserum against guinea pig immunoglobulin indicating that cytotoxicitiy was indeed due to the presence of an antibody. Perlmann interpreted his findings as representing antibody induced nonspecific cytotoxicity of normal lymphocytes brought about by aggregation with the target cells through mixed agglutination or resembling cytophilic affinity of the antibody for lymphocytes. In subsequent experiments with

guinea pig immunized with thyroglobulin this finding was again observed with chicken erythrocyte target coated with thyroglobulin. In both of these experiments, histoincompatibility as suggested by Moller was not required for cytotoxicity to be mediated since normal lymphocytes could kill an autologous target in the presence of antiserum (136).

MacLennan (107) did his study in this system with Chang liver cells (a malignant human cell line) as target exclusively, with antisera from rats and rabbits and cells from rats and human. It was later shown to be an extremely sensitive system, at least 1000 times more sensitive than complement mediated lysis (108). The antibody was found to be in the IgG fraction (108) and the cells, non T-cells (more discussion on the cells involved in Chapter II).

3. Terminology

Because of its potential biologic importance, many laboratories became interested in this in vitro phenomenon, each with its own designation for the test system. The most appropriate terminology appears to be "antibody dependent cellular cytotoxicity" or ADCC as used by Fahey and his associates (200). Wunderlich calls it "lymphocyte dependent antibody" or LDA (196). Ceppellini calls it "lymphocyte antibody lytic interaction "or LALI (173). In this thesis, it is called "antibody mediated cell dependent immune lympholysis, or "ABCIL", because it is mediated by antibody and the target cell damage is dependent on lymphoid cells. However, not all investigators working in this field agree that it is the lympho-

cyte that kills the antibody coated target, and it will become evident later in this thesis that not all experimental models are strictly "lymphocyte dependent". The targets used throughout this study were almost exclusively human peripheral blood lymphocytes, hence the term "lympholysis".

4. Kinetics and Mechanism

The antibody and the cells involved in this test system will be discussed in more detail later in this thesis. Suffice it to say here that this is a very efficient mechanism in producing cell destruction. The system works efficiently with antiserum diluted in high titres (60, 81, 108, 123, 136). Compared with the complement dependent lysis of target cells the reaction occurs much more insidiously (136), yet it is a thousand times more sensitive. It is temperature dependent and the lysis can be totally inhibited at low temperature (149). Close contact between the effector and the target cells is a prerequisite (12, 60, 137, 149) and this is accomplished first of all by the $F(ab)_2$ of the antibody molecule attaching to the antigenic determinants on the target. It is through binding of the $F(ab)_2$ to the target that some conformational change takes place and the Fc become available to bind with the Fc receptors on the effector cells (54, 123). (In this context, the antibody in this system is not a cytophilic antibody (88) which can bind with macrophages through its Fc without prior sensitizing the target cell). Removal of the Fc portion of the antibody molecule, therefore, prevents the cytotoxicity (60, 123). Following

aggregation the effector cells are activated to kill the target by an as yet undefined process. DNA synthesis or protein synthesis is not required (123, 136). Soluble mediators are not detected in the culture medium (60, 123, 137); but locally produced mediators cannot be excluded. The final event is target cell death by osmotic lysis (12, 13, 149).

5. Specificity

This immune mechanism has varying degrees of antigen specificity of the $F(ab)_2$ binding sites. For example, rabbit anti-sheep fibroblast will cause cell-mediated lysis of sheep fibroblasts but will not lyse human fibroblasts (123). Following the recognition step, target cell destruction by non-sensitized lymphocytes is non-specific. Thus, antibody treated target cells can be lysed by autochthonous lymphocytes (136).

6. Conclusion

Antibody induced lymphocyte mediated cytotoxicity is a newly established *in vitro* immunologic phenomenon resembling phytohemagglutinin induced lymphocyte mediated cytotoxicity (85). Following aggregation to the target cells, normal lymphocytes become non-specifically cytotoxic and kill the target by an unknown process. Because of the sensitivity of the system in mediating cytotoxicity, it is believed to have *in vivo* biologic significance. Thus, it has been postulated to have a role in the defense mechanism against

viral infections (151), tumor (74, 191) and leukemia (19, 82), and in the pathogenesis of certain autoimmune diseases (20, 53). The role of this type of immune mechanism in the field of kidney transplantation has not been fully delineated, though it had been claimed to be responsible for an acute irreversible rejection (159). This will be discussed in more detail in a later chapter.

At this point it is necessary to digress from consideration of the main immunologic system used in this thesis to review two different subjects, namely, histocompatibility and tissue sensitization. This in vitro antibody-induced cell-mediated cytotoxicity system has been applied to the study of allosensitization, and to detection of histocompatibility antigens. The auther's research in these areas are presented in later chapters.

C. The major histocompatibility system in mouse and man.

The term "histocompatibility antigen "was first introduced by Snell in 1948 (155) and was used to denote antigens on cells and tissues capable of inducing an immune response in the host after grafting leading to the rejection of the grafted tissue. The rapidity of primary rejection depends on the "antigenic strength" on the graft which is absent in the host. These histocompatibility (or transplantation) antigens can be grouped into histocompatibility systems; antigens that can induce an immune response in the host and cause graft rejection in a short time are said to be "strong" and belong to a "major" system (33), in contrast to those "weak" antigens of a "minor" system which cause graft rejection in longer periods of time. In all mammalian and avian species studied to-date, there is a "major" histocompatibility system (Appendix A). In mammals, the H-2 system in mice and the HLA in human have been studied in greatest detail and will be further discussed.

1. The H-2 system in mice

The history of the H-2 system began at the turn of the century with the advent of tumor transplantation. Tumors of the Japanese waltzing mouse could be successfully transplanted into mice of that inbred strain but not to other strains (a similar result was also observed in the "white" mice which had achieved a high degree of inbreeding). When this tumor was propagated in succeeding generations of this inbred strain crossed to other unrelated strains, Tyzzer (174) observed an intriguing finding: F_1 hybrids between the Japanese waltzing and common laboratory mice were susceptible to tumor growth, but not F_2 or the F_3. This paradox to simple Mendelian genetic law was explained by Little (102) who proposed a genetic theory of tumor transplantation in which susceptibility to a tumor transplant is determined by several dominant genes. The exact number of genes involved can be estimated from the formula $f = (3/4)^n$, where f is the fraction of F_2 mice dying from the tumor and n is the number of susceptibility genes. This postulation was later verified experimentally (103) and the number of susceptibility genes were estimated to be 12 - 14 even though the nature of these tumor susceptibility genes remained undefined.

It was originally believed that tumor cells differed from normal cells and the defense mechanism against tumor was directed against this difference. Thus, it was difficult to explain why tumor transplanted within an inbred strain of mice could not be rejected.

An alternative explanation was proposed by Haldane (75) who postulated that immunity was directed against alloantigens, like blood group antigens, rather than tumor specific antigens, and that these alloantigens induced an immune response in hosts which lacked them. Tumor transplanted within an inbred strain do not induce such a response because the recipient of the tumor share the same antigens with the donor.

It remained for Gorer to establish that such antigens exist in mice. Using rabbit anti mouse antiserum (64), he was able to detect 4 blood group antigens I, II, III and IV. Antigen II was present on A but absent on C57BL cells. Using an A strain tumor in grafting experiments onto A, C57BL, and F_1 and F_2 hybrids of these two strains (65), it was found that the A strain and the F_1 were susceptible while the C57BL strain and half of the F_2 were resistant to the tumor. All the susceptible and some of the resistant F_2 mice carried antigen II. Thus, susceptibility to this tumor appeared to be determined by two or more genes, one of them was identical with the gene coding for antigen II. In addition, sera of the C57BL mice that had rejected the tumor contained hem-agglutinating antibody against strain A red cells, identical to that of the rabbit anti-II antiserum (66) thus establishing antigen II as an alloantigen. Such antigens were later designated by Snell as histocompatibility antigens and the genes coding for these antigens, histocompatibility genes (H-genes). The H genes coding for antigen II were designated as H_2 (67) and were later changed to H-2.

Through Snell's ingenious approach towards studying histocompatibility systems in mice by producing many congenic lines differing at only one histocompatibility locus, it was found that an H-2 difference is much more effective in causing graft rejection than non-H-2 differences. It was also discovered later that the H-2 locus was, in fact, not a single locus but consists of a set of loci (156). The H-2 then became known as the major histocompatibility complex of the mouse.

As it stands today, the H-2 complex on chromosome number 17 (94) is divided by the Ss locus (152) into two ends, the K-end (towards the centromere) between H-2 K locus and Ss, and the D-end (towards the telomere) between Ss and H-2D. It is further subdivided into 4, or possibly 5, regions, K, I, S, "X", and D, each consisting of at least one defined locus and a number of undefined loci. The I region has been found to consist of 3 subregions, IA, IB, and IC, each contains at least one locus. With the exception of the S region, defined loci in all other regions are responsible for genetic control of graft rejection and/or lymphocyte activation in mixed lymphocyte culture; and the I region is also responsible for genetic control of immune response to antigens (see Appendix B)

2. <u>The major histocompatibility complex in man.</u>

Obviously, the approach of histogenetic study in mice cannot be applied to human; and it is unrealistic to think that the same degree of sophistication can be achieved in the study of histocompatibility systems in man as it has been in mice. For a time it

seemed unlikely that histocompatibility phenotypes and genotypes could ever be analyzed in man as it had been in mice. Despite all the practical limitations the MHC in human is better studied than that of any other mammalian species, except the mouse, to-date.

a) The LA and "FOUR"

The history began in the early 1950's with the finding of a French scientist, Jean Dausset (34), that serum from patients who had been multi-transfused contained antibodies capable of causing clumping of leukocytes, independently of erythrocyte antigens, from some unrelated individuals but not from the serum donor. It thus appeared that blood transfusion had stimulated the production of these leukoagglutinating antibodies in those patients. Not long afterwards, it was discovered that such antibodies could also be produced in women following pregnancy (132, 185), against antigens present on leukocytes of the husbands. The first leukocyte antigen was named by Dausset, in 1958 (35), after the initials of the first three subjects in whom the antigen was identified---antigen MAC (or Mac). Then in 1959, van Rood described two new leukocyte antigens 2 (186) and 3 (187).

The first leukocyte group system was described by van Rood in 1962 (182). In his study, a large panel of multiparous sera were tested against a large panel of leukocytes from unrelated individuals. With the help of a computer analysis he was able to group sera according to the agglutination patterns into groups. One group (3 sera) gave agglutination patterns which were significantly similar, though not identical, to part of the panel cells. Another group

(5 sera) also gave a reaction pattern against panel cells in a highly similar fashion but with a contrasting distribution to the first group. Using these two groups of sera, the Dutch population could be shown to carry either group of antigens on their leukocytes, or to carry both. By family segregation study, the genes responsible for the expressions of these two groups of antigens were found to be inherited as simple Mendelian autosomal co-dominant alleles. Since 3 antigens had already been described - MAC, 2, and 3, van Rood named these two groups of antigens the "4" system; one 4a and the other 4b.

Two years later, Rose Payne and her associates in Los Angeles, described another new leukocyte system (133) which, at that time, consisted of two allelic antigens LA 1 and LA 2 (LA for Los Angeles). This LA system, with the third antigen found in 1966 (LA3), was thought to differ from van Rood's 4a - 4b system (15) as supported by a single family study in which the antigens of the two systems segregated in an independent fashion.

With additional descriptions of other new antigens and with different designations made by various workers, the whole field appeared extremely confused and complicated. Then, in an international workshop held in Leiden, Dausset (37) proposed a unifying system called Hu-1 (for Human -1, as compared to Mouse H-2 system). Ten defined leukocyte antigens were described, characterized by a very high frequency of positive and negative associations with one another suggesting that these antigens might be components of a

complex immunogenetic system. This was supported by the results of another workshop, 2 years later, which showed that most of the leukocyte antigens were determined by closely linked genes at one chromosomal region; and that antigens of the LA series of Payne and the "FOUR" series of van Rood might be determined by genes at two different sites within this chromosomal region (25). This was later confirmed (1). The Hu-1 system was changed to HL-A (for human leukocyte, group A) locus or region; the LA and "FOUR" system were called LA and "FOUR" series, or the first and second sublocus of the HL-A; and the antigens were indicated by a number following the symbol of the system. Thus, HL-A1, HL-A2 and HL-A3 belonged to the LA series; and HL-A5, HL-A7 and HL-A8 belonged to the "FOUR" series; and so on. To avoid confusion, it was agreed not to use the designation HL-A4 for any HL-A antigens.

b) The AJ

The existence of a third locus in the HL-A region was suggested by Sandberg in 1970, (145) based on absorption and family segregation studies using the complement dependent cytotoxicity test. This was later confirmed and designated AJ locus according to the original description of the serum named after the woman AJ (157).

The gene products of these three closely linked HL-A loci are expressed on most tissues and cells including lymphocytes. They can be detected by two serological techniques - leukoagglutination and lymphocytotoxicity. For the sake of convenience, Bach used the term "serologically detectable" or S.D. (6) determinants

interchangeably with these antigens to distinguish them from another category of gene product determinants also present on lymphocytes but not readily detectable by these serological methods, though they can be defined by another laboratory technique called mixed lymphocyte culture test (MLC). These latter determinants have been called MLC gene products, lymphocyte activating determinants (LAD or Lad) (56) or simply lymphocyte-defined (L.D.) factors. The S.D. - L.D. terminology has been widely used, though it now seems inappropriate in view of the fact that these Lad's can now be detected serologically and the so called S.D. determinants may have lymphocyte activating property in MLC.

c) The MLC locus

When lymphocytes from two genetically non-identical individuals are cultured together in vitro, a proliferative response is observed (5, 9). The cell proliferation can be assayed by direct enumeration of blastoid transformed cells, or by measuring the incorporation of radioactive thymidine. The response of each lymphocyte population can be studied separately by doing the one-way or unidirectional MLC (UMLC) test. This is done by treating the stimulating cell population with irradiation or mitomycin C, which inhibits cell proliferation without interferring with the stimulating capacity.

The cells that respond in MLC are mainly T-cells (thymus derived) though some B-cells (Bone marrow derived) are also capable of responding (3). Contrary to previous belief in a T-cell stimulator, recent

studies indicate that B-cells are the stimulating cells in MLC (29, 139, 140, 193).

In mice, H-2 incompatibility at the K end is sufficient for MLC stimulation (143). Sometimes, this can also be produced by the D end difference (96). In addition a non-H-2 linked locus (the M-locus) can also provoke a MLC response (55). In Man, HL-A identical sibling pairs are usually non-stimulatory in MLC whereas unrelated individuals and HL-A non-identical siblings are. This led to the initial belief that MLC interaction was governed by the same loci as those determining HL-A antigens (2). However, cells from HL-A identical unrelated individuals do stimulate each other in MLC (112) and recently, cells from some HL-A identical sibling pairs have also been shown to cause reciprocal stimulation (197). Further, MLC experiments in families with recombination between the first and second HL-A loci have shown that sibling pairs who, because of recombination were only incompatible for one first series HL-A antigen, did not stimulate each other in MLC, whereas one second series incompatibility led to stimulation (51, 198). These findings suggest that MLC interaction is caused by disparity at another locus (or group of loci) separate from but closely linked to the second locus of the HL-A (48, 198). The gene products of this locus are expressed on the lymphocyte surface but not readily detectable by conventional serological means.

However, it is now possible to type for some of the MLC gene

products in unrelated individuals using the so called MLC homozygous cells from individuals who inherit an identical MLC gene from each parent (49, 112, 178). Such individuals will not stimulate either of their parents, nor will they stimulate other unrelated persons carrying the same MLC gene, in one way MLC tests. Increasing numbers of MLC homozygous cells are being identified and it is very likely that typing of these MLC gene products will become possible in the near future.

Recent findings suggest that there might be another MLC locus in the region of the HL-A responsible for weak stimulation. This "minor" MLC locus, if it exists, is close to the first HL-A locus (52, 169).

d) Present view of the human MHC and nomenclature

It is now known that the human MHC is located on chromosome no. 6 (98). Within or linked to this chromosomal segment are several other loci, one of which, the Gb locus (161) controls the serum level of glycine - rich B-glycoprotein (GBG) which is part of the human complement system. Recombination studies suggest that this locus is linked closer to the second than the first HL-A locus. Since the S region of the H-2 in mice is also involved in the control of the complement system (40), the localization of this Gb locus within the HL-A further suggests homology in genetic organization of the mouse and human major histocompatibility system (Appendix C).

After the 1975 international histocompatibility workshop, new

terminology was adopted by the WHO Committee on leukocyte nomenclature. The MHC in human is now called HLA for Human Leukocyte Antigen (the hyphen was dropped). The four loci are now referred to as locus A (previously first, or LA), B (previously second, or "FOUR"), C (previously third, or AJ) and D (previously LD, or MLC). The antigens of these loci are designated HLA-A, -B, -C or -D followed by a number if such antigens have an internationally accepted specificity. Following the previous practice, antigens with provisional specificity are identified by a number preceded by the letter W (for Workshop); the W will be dropped when the specificity is approved by the committee.

A complete list of these loci and antigens are presented in Appendix D.

e) Relevance of the MHC in transplantation

Apart from the ABO blood group system (24, 62) the HLA (A & B) was considered to be the only major transplantation antigenic system in human. Evidence in support of this notion had been obtained by several groups of investigators in kidney transplantation and skin grafting experiments in genetically related individuals (7, 26, 99, 188). However, in unrelated individuals no significant correlation could be established between the number of HLA (A & B) antigens, compatible between donor and recipient, and the skin graft surivial time (26, 188). Similar findings have also been obtained in clinical transplantation in North America

(11, 99, 116), but not in Europe (38, 57, 126). This lack of correlation between HLA (A & B) compatibility and graft results in unrelated individuals, in contrast to the good correlation in siblings, suggests that another locus, closely linked to HLA (A & B) may be more relevant to transplantation than HLA (A & B). In siblings, HLA (A & B) compatibility might, unknowingly, also match for this locus, hence prolonging graft survival, while in unrelated situations, matching for this locus in most instances could not be achieved despite compatibility for HLA (A & B). In favour of this locus being HLA-D is the accumulating evidence that suggests an association between the degree of MLC stimulation or MLC (HLA-D) compatibility and graft outcome (30, 57, 79, 167, 184, 189). It thus appears that the good graft results obtained in HLA (A & B) identical sibling may be due to HLA-D identity because of the close linkage between these loci and at the present one of the major efforts that many transplantation centres are making is to make it possible to type for these HLA-D antigens in unrelated individuals.

f) Tissue typing techniques

As mentioned earlier, HLA antigens of the A, B and C loci are detected serologically. The principles of the two most widely used techniques, leukoagglutination and lymphocytotoxicity were originated by Gorer for H-2 serologic study (68, 69). Leuko-agglutination was used almost exclusively by earlier workers;

lymphocytotoxicity was adopted later (162). Both techniques have been miniaturized and modified (121, 166). Other not as widely used techniques include platelet complement fixation of Shulman (153), mixed agglutination of Milgrom (117), immune adherence of Melief (111), etc.

Determination of the HLA-D antigens of a defined specificity is still in the early stage of development. The most reliable method for this purpose at present is by doing MLC tests using HLA-D homozygous cells. Six workshop specificities have so far been identified (Appendix D) though many more are being characterized. With the present speed of development, it is expected to have nearly all specificities defined in the very near future.

The technique of antibody-induced cell-mediated cytotoxicity has been used in the study of allosensitization in human, following pregnancy (43, 109, 173) or after blood transfusion (81, 101). Compared with lymphocytotoxicity, this technique appears to be more sensitive and capable of detecting antibody specificities that the former fails to detect (81, 173), thus raising the possibility for this technique to detect non-HLA (A & B) antigens.

The main task of this study was to explore further the nature of these non-HLA (A & B) specificities. This will be presented in Chapter III. As it turns out, this technique is capable of detecting HLA-D specificities which other serological techniques cannot detect. The effector cells are crucial in doing all these

studies and some interesting data on activity of effector cells from different individuals will be presented in the next chapter. The significance of this type of antibody in the field of transplantation, a highly controversial subject, will be discussed in Chapter IV. Some speculations and clinical applications will be made in Chapter V.

Chapter II. The Effector Cells Activity (ECA) in Antibody Mediated Cell Dependent Immune Lympholysis

A. Introduction

Even though the first description in the antibody-induced cell-mediated cytotoxicity has been made no less than 10 years ago (122), there is still considerable debate on the specific types of cells involved in the lytic process. Various authors have independently shown, in different animal models of this system that T-cells are not involved (14, 80, 105, 176) but there have been various conclusions as to which class of non-T lymphocytes, or macrophages, are principally involved as effector cells. One problem inherent in this test system is that different investigators use different models. Therefore, it is not surprising that they reach different conclusions. In this chapter, the author intends to show, using two different antibody - target systems, that different types of cells are required to lyse these antibody sensitized targets in human. In addition, evidence will be presented that in normal individuals there is a difference in the activity of their lymphoid cells to lyse antibody coated target lymphocytes, suggesting that sex hormones may have some influence on the effector cell activity (ECA) in this particular target system.

B. Literature review

1. Evidence that T-cells are not the effector cells in antibody-induced cell-mediated cytotoxicity.

With the exception of Fakhri and Hobbs who claimed that T-lymphocytes are the killer cells in their model of rat versus mouse plasmacytoma tumor (54), it is generally agreed that T-cells are not involved in this system.

Using his Chang cell target system with rat anti-chang serum, MacLennan and his associates (80) have shown first, that cells from unsensitized rat's thoracic duct lymph did not have any cytotoxic activity against the antibody coated chang cells. Secondly, they showed chronic drainage of cells from the thoracic duct depleted rat spleens of cells that were responsive mitotically to PHA. Finally, the remaining cells in the depleted spleen were shown to have enhanced cytotoxic activity against the antibody-sensitized target. Taken together, this argues strongly against T-cells being the killer cells in this antibody-induced cell-mediated system.

In a later experiment (105), rats were either thymectomized, sublethally irradadiated, or subjected to both these procedures. All of these manipulations progressively impaired the mitogenic response of splenic lymphocytes to PHA, yet their cytotoxic capacity remained intact. It was concluded that effector cells are independent of the thymic influence.

Since they did not find macrophages to be cytotoxic in their system, effector cells were thought to be a subpopulation

of B-cells distinct from antibody producing cells, which they called cytotoxic "B" cells (104).

Similar conclusions have been reached by van Boxel and his associates in their study in mice (176). They used guinea pig antiserum against burro erythrocytes with killer cells from CBA mice which had been thymectomized, lethally irradiated and reconstituted with syngeneic bone marrow cells. In other experiments, spleen cells from BALB/c mice were treated with anti θ and complement to eliminate T-cells before being used in the cytotoxic assay. The results in both sets of experiments were not different from their controls. In fact, spleen cells from thymus deprived animals were more effective than those from untreated mice. To investigate the role of B-cells in their test system, they treated spleen cells with anti-light chain antibody with or without complement; in both cases there was diminished cytotoxic effect. No firm conclusion was drawn but it was suggested that either B-cells or a subpopulation of B-cells were the effector cells.

Evidence against T-cells as the effector cell population was obtained by Blaese and his associates (14), who compared peripheral blood lymphocyte preparations from several groups of immunodeficient patients as effector cells in their test system in which anti-HLA antibody was used against human leukocyte target. In these human studies the same conclusion was reached. Specifically, peripheral blood lymphocytes of several patients with Wiskott-Aldrich syndrome and several cancer patients (judged to have

deficient T-cell function because of their impaired lymphocytic response to allogeneic stimulation in MLC, to specific antigens or to non-specific mitogenic stimulation) had normal effector cell activity. The third group of patients was most interesting. These were patients with intestinal telangiectasia who were chronically depleted of recirculating small lymphocytes through leakage into their bowel. Their peripheral blood lymphocytes had enhanced killer cell activity but at the same time they were severly impaired in their blastogenic response to all three stimuli just mentioned. Further more, lymphocytes from their chylous pleural fluid exhibited exactly the opposite phenomenon - they had impaired effector cell activity with enhancement in their proliferative response. A fourth group of patients with hypogammaglobulinemia had clearly diminished cytotoxic capacity as a group. However, there was no good correlation of the number of immunoglobulin-bearing cells with their cytotoxic capacity. Nevertheless it was concluded that the effector cells belonged to a subpopulation of B-cells or another class of non-T-lymphocytes.

2. Evidence against immunoglobulin-bearing cells as effector cells in antibody-induced cell-mediated cytotoxicity.

In addition to the studies cited above, Perlmann (138) also made the same conclusion that B-cells were the cytotoxic effector cells in a heterologous system employing human effector lymphocytes, chicken erythrocyte (CRBC) target, and rabbit anti-CRBC serum. By

passing normal human lymphocytes onto a glass-bead column precoated with human IgG and rabbit anti-human Ig, the B-cells were removed; at the same time, cytotoxicity was markedly reduced. This was taken as evidence that Ig-bearing cells were cytotoxic cells.

Using a different method to deplete Ig-bearing cells, Wisloff and Froland (195) came to an exactly opposite conclusion in the same test model of CRBC + rabbit anti-CRBC and human lymphocyte effector. The B-cells were removed by passing the lymphocyte suspensions onto a nylon wool column instead. Despite the depletion of B-cells as judged by immunofluorescent staining, cytotoxicity was enhanced. Similarly, several hypogammaglobulinemic patients lacking B-cells had intact effector function.

It is possible that the Ig-anti-Ig column in the former study (138) removed not only the Ig-bearing cells but also other Fc receptor-bearing cells responsible for antibody-induced killing, accounting for the discrepancy in their results. Thus, it appeared that B-cells were not involved in this system in human. In support of this notion is a study done by Greenberg in mice (72) which showed that this antibody-dependent effector was neither an Ig-bearing cell nor a T-cell. He called it a "null" cell. In a later study (73), this cell was further defined by its cell size, morphology and adhering property as belonging to the monocyte series.

Finally, in a heterologous system using chicken lymphoid

effector cells, bursectomy, while severely depleting Iglevels in the chicken, failed to abate the effector cell function (21).

Therefore, it is safe to conclude that B-cells are not involved in this antibody dependent cytotoxic process.

3. Evidence that there may be more than one population of effector cells.

At this point, it should be pointed out that in many of the studies done, the phagocytic cells had been intentionally left out to eliminate the role of phagocytosis as an effector mechanism. Phagocytosis can play a role, albeit minor, as shown by Perlmann (137). Holm had also shown, using human AB erythrocytes and human anti-AB sera that monocytes and granulocytes killed these antibody-sensitized erythrocytes mainly by phagocytosis, while lymphocytes had no effect (83). These phagocytic cells can also kill by a process independent of phagocytosis and the presence of these cells in lymphocyte preparations can often speed up the initial rate of lysis (137, 192).

That there are more than one effector cell population in this antibody dependent cytotoxic system was clearly documented by Gelfand (60) whose experimental model was CRBC, rabbit anti-CRBC and cells from different lymphoid organs of rabbit as effector. When the spleen cells were used, there was an initial rapid phase of lysis, followed by a more gradual phase. This rapid phase activity disappeared when the spleen cells were cultured

for 4 hours prior to adding the target and it was absent from the lymph node cells and thymus cells. It appeared that there are at least two populations of effector cells in the spleen. Addition of phagocytic cells from the peritoneal cavity to the lymph node cells produced the two-phased pattern of the spleen cells. So macrophages (and presumably granulocytes) and lymphocytes both played a role in his particular test model. Similar conclusions had been reached by another group of investigators in mice, using EL-4 leukemic cells as target (200).

In these studies, as well as others, macrophages could clearly kill target cells in the presence of specific antibody. However, in other studies, macrophages could not kill (43, 104, 123, 199). It appears likely (1) that different types of effector cells may be required to lyse different targets; (2) that cells of the monocyte-macrophage system or the adherent cells are capable of lysing antibody-coated erythroid cells and this can be accomplished through a process independent of erythrophagocytosis (12); (3) that these adherent cells may not be capable of lysing non-erythroid target cells although there are reports that suggest the contrary (41, 200); and (4) that the non-T-lymphocytes are required for the lysis of these non-erythroid targets. Whether or not these non-T-cells are also capable of lysing antibody coated erythrocytes remains unclear.

C. Materials and Methods

1. Experimental models.

Two different human target systems, lymphocytic and erythrocytic, are used in this study.

a) The lymphocyte target system. Women after 3 to 4 pregnancies usually develop ABCIL antibody in addition to other antibodies. Thus, sera from multiparous women are a good source of antibody against their husbands' peripheral blood lymphocytes as target cells. It is, therefore, very convenient to use this wife-vs-husband model in this study.

Antiserum. In these experiments except for data in Table 3, serum from one multiparous woman (HLOC) was used. HLA profiles of this woman and her husband are A2, A3, B14, BW35 and A3, A11, B7, B12, respectively. In this serum, complement-dependent cytotoxic antibody was directed against B12, whereas ABCIL activity was much more widespread and was clearly not restricted to the HLA antigens of the husband either directly or by HLA cross-reactivity as defined by Mittal and Terasaki (120). However, the major ABCIL activity may also be directed against B12 (see results in Chapter III).

Target cells. In most experiments, peripheral blood lymphocytes from one individual (JHAY) with HLA-A11, A32, B12, and BW35 were used with HLOC serum. This particular target has previously been shown to be equally susceptible to lympholysis in the presence of

HLOC serum as the husband's lymphocytes.

b) The erythrocyte target system. Human group A or B erythrocytes are susceptible to lysis in the ABCIL system in the presence of the commercially available human (Hyland, Costa Mesa, Calif.) or rabbit anti-A or Anti-B antiserum (Ortho Diagnostic, Don Mills, Ont.). Although the agglutinating activity of these antisera from different batches is approximately equal, the ABCIL activity against RBC varied from batch to batch. However, it was found that 1:10 dilution was satisfactory for different batches.

2. The effector cells.

A total of 52 presumably normal individuals were used in the lymphocyte target system. Of these, 9 were pediatric patients seen for dental caries, epilepsy, congenital heart disease or inguinal hernia, with no obvious infections or hematological disorders. Their ages ranged from 11 months to 12 years. Twenty-seven were laboratory personnel, doctors or nurses, all allegedly in good health with ages ranging from 16 to 63. Finally, 16 senior citizens in two nursing homes volunteered in the participation of this study. Their age ranged from 67 to 92. A few had diabetes mellitus but none had any infection at the time of study. In the erythrocytic system, only the 27 normal hospital personnel mentioned above were involved in the initial study; 14 of these were males (aged 16 - 48), and the other 13 were females (aged 21 - 49).

3. Preparation of the effector and target cells.

This was modified from the method used by Boyum (17). Peripheral blood was collected into 7 to 10 ml heparinized tubes which were centrifuged to separate the cells from plasma. About 1 ml of the buffy coat was resuspended with 1 ml of Hanks' balanced salt solution and layered on 2.5 ml of Ficoll-isopaque (sp. gr. 1.077) in 10 x 75 mm glass tubes. These were then centrifuged at 1500 rpm from 20 min. The lymphocyte layer at the junction was then transferred to a plastic tube, washed, and excess erythrocytes lysed with distilled water. After one more wash, the lymphocytes were finally resuspended in medium 199 with 10% fetal calf serum (FCS). This method regularly gave a lymphocyte yield of about 80% with 15 - 20 large mononuclear cells and less than 5% polymorphonuclear cells, and a viability of greater than 98%. The effector cells were adjusted to 2 x 10^6/ml. Ten million target cells in 0.1 of medium 199 with 10% FCS were labelled with 50 to 100 u ci of ^{51}Cr as sodium chromate (Amersham, Toronto, Ont.), washed four times and finally resuspended in 5 ml of medium 199 with 10% FCS.

For preparation of the erythrocyte target, 0.1 ml of heparinized blood from an A or B individual was washed in medium 199 twice and then made into an erythrocyte suspension of roughly 200 - 500 x 10^6/ml in medium 199 with 10% FCS. One-tenth of 1 ml of this was similarly labelled and washed as above and finally resuspended in medium 199 with 10% FCS at 4 x 10^6/ml.

4. Procedure for comparison of effector cell activity (ECA) in both ABCIL systems.

In all experiments that ECA was being compared, the two ABCIL systems were carried out concurrently using identical effector cell suspensions for both systems.

To each 10 x 75 mm glass tube, 0.5 ml of various effector cell suspensions were installed, followed by 0.025 ml of target cell suspension, thus, making the effector to target ratio of 20:1 (unless otherwise stated) for the lymphocyte target system. In the erythrocyte target system, varying the effector to target cell ratio over the range of 40:1 to 10:1 did not change significantly the amount of ^{51} Cr release in the presence of specific antiserum; and the 10:1 ratio was used in most experiments in this study. Finally, 0.05 ml of heat inactivated specific antiserum was added (FCS for the controls). After mixing, the tubes were incubated at 37° C in 5% CO_2 atmosphere for 3 to 4 hours, then 2 ml of cold Hanks' balanced salt solution was added and the supernatants were separated from the cell pellets after centrifuging at 4° C at 1500 rpm for 7 min. ^{51} Cr release was calculated from:

$$^{51}\text{Cr release (\%)} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant + cpm in cell pellet}} \times 100$$

and specific ^{51} Cr release from:

$$\text{specific } ^{51}\text{Cr release (\%)} = \frac{\text{experimental } ^{51}\text{Cr release - background } ^{51}\text{Cr release}}{\text{maximal } ^{51}\text{Cr release - background } ^{51}\text{Cr release}} \times 100$$

Maximal ^{51} Cr release from lymphocyte target by repeated freezing and thawing in liquid nitrogen was about 70 to 80%. Background release in the presence of FCS was from 1 to 2% per hour. For ^{51} Cr labelled erythrocytes, background release was usually less than 1% per hour and maximal release 90 - 98%. Variations between duplicates seldom exceeded 3%.

5. Depletion of the adherent cells.

In a number of experiments, Ficoll-isopaque separated cell suspensions were depleted of adherent cells either by allowing them to stick to a plastic culture dish, as described below, or by means of a glass bead column. Four to 5 ml of fine gum acacia coated glass beads (size 0.2 mm per bead) were placed in a plastic straw which was plugged at one end with nylon cloth. Two milliliters of effector cell suspensions ($5 - 10 \times 10^6$ cells/ml) were allowed to stay in the glass bead-filled straws for 10 min. Then the cells

were eluted from the column with excess of medium 199 with 10% FCS, washed twice, and resuspended in medium 199 with 10% FCS.

6. Preparation of the adherent cells. In some experiments, effector cell suspensions (5 - 10 x 10^6 cells/ml) were allowed to stick to a plastic tissue culture dish at 37° C for 90 min. The non-adherent cells were then carefully removed and the Petri dish was carefully washed once with medium 199 with 10% FCS. The adherent cells were then recovered by gentle scraping with a rubber policeman, washed once, and resuspended in medium 199 with 10% FCS.

7. Irradiation of the effector cells.

In a few experiments the Ficoll-isopaque separated cells were subjected to irradiation from a 660 keV ^{137} Cesium source, at a distance of 15 cm from the source and an output of 226 rads/min. These irradiated cells were then washed once and resuspended in medium 199 with 10% FCS and used immediately.

D. Results

1. Results showing variability of ECA among normal individuals, in the lymphocyte target system.

a) Comparison of ECA of 52 persons, lymphocytes each studied on one occasion. ECA expressed as the percentage of specific ^{51}Cr release from JHAY target cells in the presence of HLOC serum and effector cells from 52 normal individuals (plotted in Fig. 1) appears to vary widely regardless of sex or age. Upon statistical analysis, however, significant differences do exist among them (Table 1). Although there is no difference in ECA between males and females in the age groups under 12 and over 63, females in the age range of 16 to 49 have significantly lower ECA than males in the same age range ($p<0.01$). Their ECA are also significantly lower than those of females over 63 ($p<0.005$). On the other hand, there is no significant difference between the ECA of males over 63 and of those in the age range 16 to 49. ECA of both of these groups, however, is significantly higher than that of males under 12 ($p<0.05$). Finally, individuals over 63 have significantly higher ECA than those under 12, regardless of sex ($p<0.005$).

b) Comparison of ECA of lymphocytes of two individuals studied on different occasions. ECA from two individuals was compared on 20 separate occasions using HLOC serum against JHAY lymphocyte targets. The data from this comparative study are shown in Table 2 and Figure 2. In all 20 experiments, cell A consistently produced a

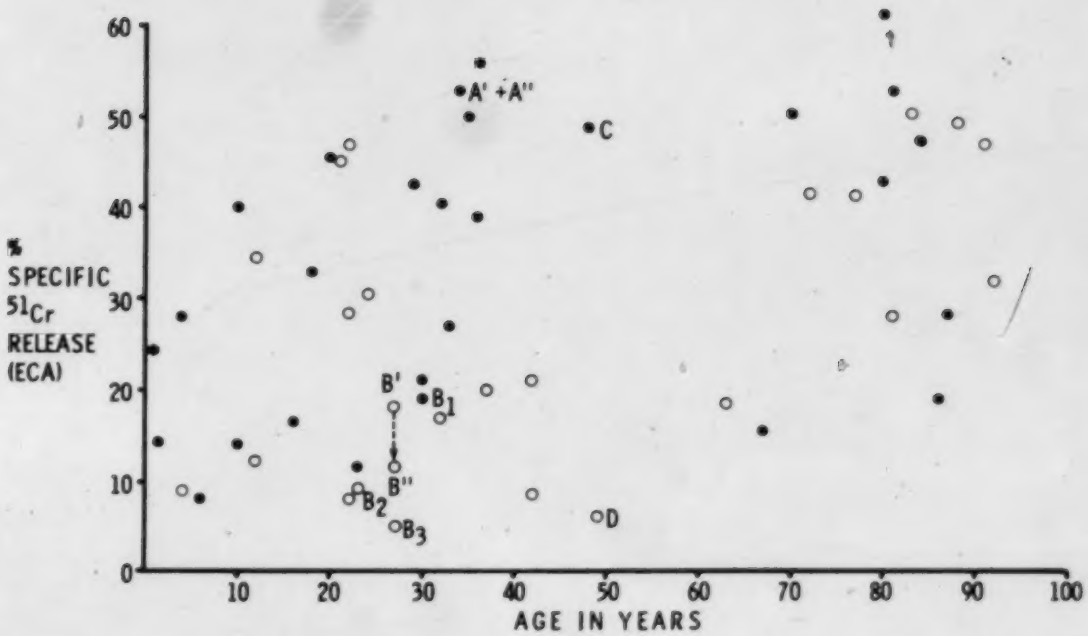


Figure 1. Percentage of specific ^{51}Cr release from JHAY target + HLOC serum as a measurement of ECA of 52 normal individuals, plotted according to age and sex. Males, closed circles; females, open circles. Data pooled from experiments done on two separate occasions with cells A and B engaged in both. ECA of cell A remain constant (from 52.5 to 52.7) whereas that of cell B fluctuated (from 18 to 11). See discussion for information on B1, B2, B3, C (HLA-A2, AW30, B8, B12), and D (HLA-A2, A11, B12, BW35).

Table 1. ECA in 52 normal individuals grouped according to age and sex (from figure 1).

Category	Sex	Age	Number	ECA[a] (mean ± S.D.)
1	Combined	Under 12	9	20.4 ± 10.9
2	"	16 - 49	27	28.2 ± 16.1
3	"	Over 63	16	39.2 ± 13.5
4	Male	Under 12	6	21.4 ± 10.6
5	"	16 - 49	14	36.0 ± 14.1
6	"	Over 63	8	39.9 ± 15.8
7	"	Total	28	34.0 ± 15.5
8	Female	Under 12	3	18.6 ± 11.3
9	"	16 - 49	13	19.9 ± 13.7
10	"	Over 63	8	38.5 ± 10.6
11	"	Total	24	26.2 ± 15.1

[a] as measured by % specific ^{51}Cr release from JHAY target (T_1) + HLOC serum after 3 hours incubation. p - values for 1 vs. 3 and 9 vs. 10 < 0.005, for 5 vs. 9 < 0.01, for 2 vs. 3 < 0.025, for 4 vs. 5, 4 vs. 6 and 8 vs. 10 < 0.05.

Table 2. Comparison of ECA's of two normal individuals, A and B[a], using the same serum and target cells, performed concurrently on twenty separate occasions.

	ECA[b]	
Experiment	cell A	cell B
1	49.6	13.0
2	56.9	26.1
3	41.1	15.3
4	43.9	15.2
5	47.9	15.0
6	54.8	18.3
7	62.7	25.8
8	52.5	22.7
9	70.1	18.9
10	46.4	14.9
11	54.4	15.7
12	60.0	22.9
13	52.7	18.2
14	52.5	11.5
15	55.4	*39.2[c]
16	50.4	16.8
17	44.3	7.1
18	52.8	17.5
19	53.0	18.5
20	57.1	26.2
mean ± S.D.	52.9 ± 6.6	18.9 ± 6.7

[a]HLA profile: A = A2, B5, BW35. B = A1, A11, B7, B8.

[b]as measured by % specific ^{51}Cr release from JHAY target (T_1) + HLOC serum after 3 - 4 hours incubation

[c]see discussion, subject not on anticonceptional medication at this time.

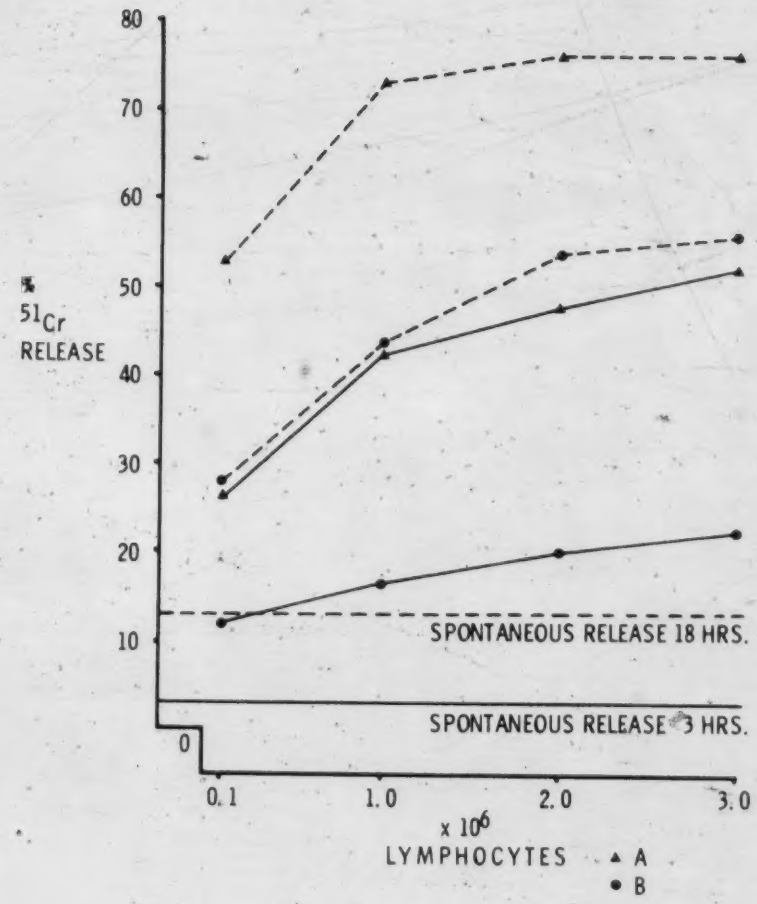


Figure 2. Percentage of ^{51}Cr release from JHAY target (constant at 50,000 cells) + HLOC serum, as caused by varying numbers of effector cells from A and B.

higher degree of target cell lysis than cell B (Table 2). In another experiment employing this same target-serum combination with varying numbers of effector cells from these two individuals, cell A still caused more ^{51}Cr release than cell B at a 30-fold lower cell dosage (Fig. 2)

Since cell B shares two HL-A antigens with the husband of HLOC (A11 and B7) whereas cell A shares none, it is possible that HLOC serum might have reacted with cell B as another target in this ABCIL system, thereby inhibiting cell B to function as fully as an effector cell as cell A. This appears unlikely because cell A and cell B, as target cells, were affected to about the same extent by HLOC serum despite the difference in number of HL-A antigens shared with the husband. Nevertheless, experiments were carried out employing these two effector cells against 5 additional target cells in the presence of 3 other multiparous women's sera. Comparison was also made between ECA of these 3 sensitized women and cell A. Again, cell A consistently caused more ^{51}Cr release from all 5 targets than cell B. Cell A also consistently caused more ^{51}Cr release than cells from the 3 women whose sera were used in the experiments (Table 3). Finally, in a preincubation study, JHAY target lymphocytes were pretreated with HLOC serum at 20° C for 1 hr. Excess serum was then removed by washing before effector cells from different individuals were added to the sensitized target. ECA of poor effector cells remained low despite the fact that no

Table 3. Comparison of ECA's of cells A and B as % specific ^{51}Cr release from different target serum combinations other than JHAY-HLOC. Comparison also made with cells from parous women whose sera used in the experiments.

Experiment	Serum	Target	ECA[a] cell A	ECA[a] cell B	ECA[a] wife's cells
1	wife 1[b]	husband 1[b]	57.7	8.6	37.0
	"	T_2[b]	52.2	1.2	26.4
	"	T_3[b]	66.4	11.5	46.0
2	wife 2	husband 2	23.0	4.2	10.6
3	wife 3	husband 3	15.0	6.4	4.3

[a] measured as % specific ^{51}Cr release from targets after 3 hrs incubation.

[b] HLA profiles of wife$_1$, husband$_1$, T_2, and T_3 are A2, A3, B7, BW15; A2, A3, B7, B14; A2, A11, B12, BW35; and A28, AW32, BW22, B14 respectively.

antiserum was present. Good effector cells, on the other hand, continued to exhibit high activity in the absence of antiserum (Table 4).

2. Comparison of ECA against antibody - sensitized lymphocytes and erythrocytes in normal individuals.

In contrast to the results seen in 1.a., above ^{51}Cr release from the erythrocyte target, in the presence of the same effector cells and rabbit antiserum, did not show any significant sex difference (Fig. 3 B). Moreover, when ^{51}Cr release by any individual's effector cells against these two targets are plotted together, there was no correlation between ECA in these two systems (Fig. 4), suggesting that different populations of cells may be required for each type of target.

3. The role of adherent and non-adherent cells in these two target systems.

By passing these cell suspensions onto the glass bead column, about one-third (20 - 40%) of the original cell numbers were removed and the small lymphocytes in the eluate became enriched to 95 - 98%. These adherent cell-depleted lymphocyte suspensions showed consistently reduced cytotoxicity to the antibody coated erythrocyte target; whereas these same lymphocyte enriched cell suspensions had enhanced cytotoxicity against the antibody coated lymphoid target cells. In contrast, the adherent cells recovered from the tissue culture dish were strongly cytotoxic in the erythrocyte target system but only

Table 4. Comparison of ECA's of different individuals in the presence and abscence of antiserum (done in parallel)

Effector cells Effector cells	ECA[a]	
	Serum present	Serum abscence
1[b]	15.5	9.1
2[c]	21.2	9.1
3[c]	50.8	24.6
4[c]	47.8	28.3

[a] measured as % specific ^{51}Cr release from JHAY target in the presence of HLOC serum, or from sensitised JHAY target with excess HLOC serum removed by washing.

[b] 23 year old female with HLA profile A1, AW31, B8, BW40.

[c] cells 2,3 and 4 same as cells B,A and C in Figure 1.

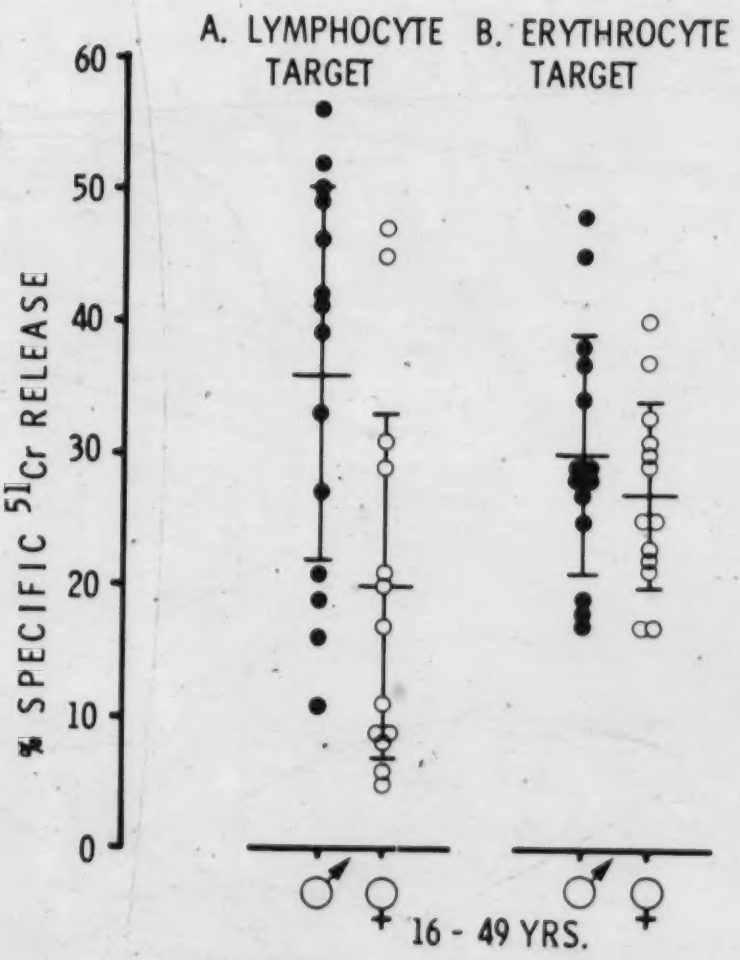


Figure 3. Activities of effector cells (ECA) from 27 normal individuals, measured as percent specific ^{51}Cr release from A, lymphocyte and B, erythrocyte targets in the presence of specific antibody and expressed as mean ± S.D. The difference in ECA by males vs. females against the lymphocyte target is significant ($p < 0.01$); whereas that against the erythrocyte target is not.

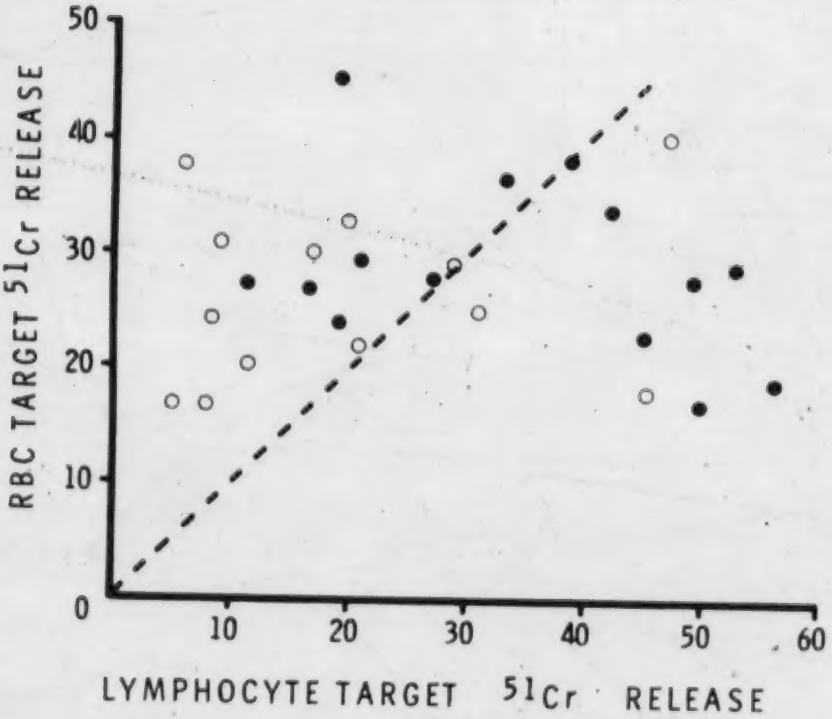


Figure 4. Scattergram showing lack of correlation of ECA from 27 normal individuals (from Figure 1.) against the lymphocyte (abscissa) and the erythrocyte (ordinate) targets.

Open circles - males, closed circles - females.

inconsistently cytotoxic in the lymphocyte target system. These findings indicate that there are at least 2 effector cell populations in lymphocyte preparations obtained by the Ficoll-isopaque separation method: adherent cells which are morphologically macrophages, and nonadherent cells or lymphocytes. The former are strongly cytotoxic to antibody-coated erythrocytes and the latter to antibody-coated lymphocytes.

The following experiments were then designed to investigate further the residual cytotoxicity of the adherent cells against the lymphocyte target, or of nonadherent cells against the erythrocyte target. This reduced residual cytotoxicity might be due to actual ECA of the less active cell type or to incomplete removal of the more active cell type in the 2 test systems.

Adherent cells were exhaustively removed by repeated passage onto glass bead column, the final lymphocyte suspensions, presumably adherent cell free, were then used in both antibody coated target systems. As indicated in Table 5, these nonadherent cells were completely devoid of any cytotoxic activity against the antibody-coated erythrocytes, although they were quite capable of lysing the antibody coated lymphocytes. At the same time, the adherent cells, recovered from tissue culture dishes, after careful removal of the nonadherent cells, were only cytotoxic to the antibody coated erythrocytes and not the antibody coated lymphocytes. These findings indicate that the residual cytotoxic effects of the adherent cells against the lymphocyte target, and the nonadherent cells against the

Table 5. Comparison of activities of ficoll-isopaque separated (F.I.), adherent (Adh.) and non-adherent (Non-adh.) cells from two normal individuals (E1 and E2) against the antibody coated erythrocyte and lymphocyte targets at different effector to target cell ratios[a].

[a] Effector to target cell ratios at effector cell numbers 0.25 x 10^6, 0.5 x 10^6 and 1.0 x 10^6 are 5:2, 10:2 and 20:2 respectively for the erythrocyte target system; and 5:1, 10:1 and 20:1 for the lymphocyte target system.

[b] % ^{51}Cr release at 0 cell number serves as the background ^{51}Cr release in each experiment.

[c] Human B erythrocytes + Human anti-B antiserum diluted 1:10.

[d] HLA(A&B) profile = A11,AW32,B12,BW35 + anti HLA-B12 undiluted.

[e] After incubation at 37° C in 5 % CO_2 atmosphere for 4 and 20 hours.

	% ^{51}Cr release[b]					
Targets		Erythrocyte[c]			Lymphocyte[d]	
Effector cells	F.I.	Adh.	Non-adh.	F.I.	Adh.	Non-adh.
E1 at 4 hrs.[e]						
0	3.7	-	-	10.3	-	-
0.25 x 10^6	26.1	35.0	3.0	25.4	9.5	42.3
0.5 x 10^6	27.9	35.2	4.5	36.3	12.7	55.2
1.0 x 10^6	26.9	-	-	47.6	-	-
E1 at 20 hrs.[e]						
0	5.5	-	-	22.0	-	-
0.25 x 10^6	62.7	68.1	7.1	72.0	25.0	85.0
0.5 x 10^6	66.7	60.0	10.2	75.9	27.4	86.1
1.0 x 10^6	55.0	-	-	77.9	-	-
E2 at 4 hrs.[e]						
0	4.9	-	-	8.5	-	-
0.25 x 10^6	17.4	26.2	2.5	28.3	8.9	36.0
0.5 x 10^6	24.3	33.4	2.0	36.4	11.5	51.0
1.0 x 10^6	29.1	-	-	45.5	-	-
E2 at 20 hrs.[e]						
0	4.0	-	-	26.3	-	-
0.25 x 10^6	53.4	59.0	3.7	74.0	25.6	79.0
0.5 x 10^6	62.5	64.2	5.0	78.2	27.3	84.4
1.0 x 10^6	58.9	-	-	78.0	-	-

erythrocyte targets were, in fact, due to incomplete removal of the other cell types.

4. Blocking of nonadherent ECA with adherent cells and vice versa.

Since both populations of effector cells carry Fc receptors, it may be functionally possible to block the cytotoxic effector of one highly purified effector cell population with the other, and vice versa, in these two systems. It was found that the adherent cells could, in fact, block the cytotoxic effect of the nonadherent cells to a certain extent against the antibody coated lymphocyte target. However, there is no evidence to suggest that the reverse is also true (Table 6).

5. Effect of irradiation.

It is known that cells of the monocyte-macrophage series are functionally radio-resistant (32, 147). It has also been shown that radiation had no effect on the effector cells, presumably macrophages, against antibody coated chicken red blood cells (41); whereas lymphocytes are quite radiosensitive. It is conceivable that irradiation of the Ficoll-isopaque separated cells may show a differential effect on their cytotoxicity against these two antibody coated target systems. Therefore, such cell suspensions were subjected to irradiation and used in both target systems. Morphologically, these irradiated cells were quite normal in appearance with no loss in cell numbers. Functionally, a discrepancy of cyto-

Table 6. ^{51}Cr release from antibody coated lymphocyte target in the presence of ficoll-isopaque separated (F.I.), adherent (Ahd.) and non-adherent (Non-adh.) cells; and mixtures of 0.5×10^6 non-adherent + 10^6 adherent cells or 0.5×10^6 non-adherent + 10^6 human ORBC.

		% ^{51}Cr release[a]				
Effector cells[b]		F.I.	Adh.	Non-adh	Non-adh. + Adh.	Non-adh. + ORBC
E1	0	5.9				
	0.25×10^6	20.2	10.3	24.7		
	0.5×10^6	29.1	12.0	35.0	→ 16.0	30.0
	1.0×10^6	38.9				
E3	0	5.9				
	0.25×10^6	21.3	5.4	27.8		
	0.5×10^6	27.9	5.8	38.2	→ 27.2	38.1
	1.0×10^6	37.3				
E4	0	5.9				
	0.25×10^6	12.0	6.6	19.2		
	0.5×10^6	16.1	7.0	25.9	→ 19.0	29.0
	1.0×10^6	19.6				
E5	0	4.2				
	0.25×10^6	18.0	5.0	26.8		
	0.5×10^6	26.4	7.3	38.8	→ 42.0	N.D.[c]
	1.0×10^6	37.3				
E6	0	4.2				
	0.25×10^6	8.8	4.3	16.6		
	0.5×10^6	9.2	5.1	21.1	→ 16.6	N.D.
	1.0×10^6	13.0				

[a] After 4 hours incubation at 37° C in 5 % CO_2 atmosphere. Background ^{51}Cr release = 5.9 % for E1, E3 and E4 and 4.2 % for E5 and E6.

[b] E1 same as E1 in Table 5.

[c] N.D. = not done.

toxic activity against these two targets was found. In the lymphocyte target system, ECA was partially suppressed at 3000 rads and at 5000 rads, it was completely abolished. This latter dosage, however, had no effect on ECA in the erythrocyte target system (Fig 5). In fact, even at 10,000 rads, ECA was not impaired in this system. This finding conclusively supports the earlier result that the macrophages are the cytotoxic cells in the erythrocyte target system but they are not cytotoxic against the antibody coated target lymphocytes.

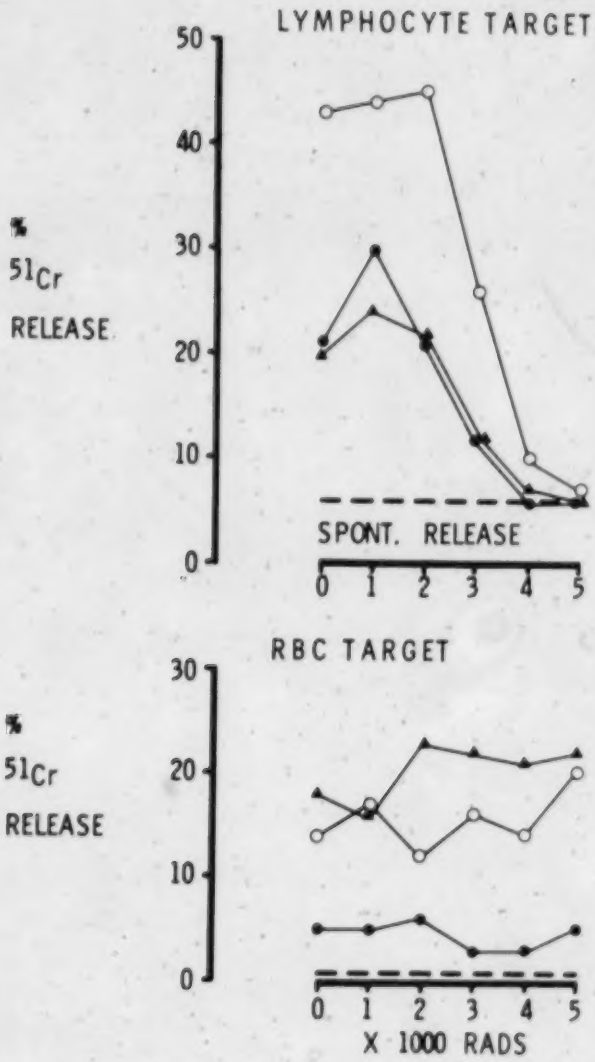


Figure 5. Effect of irradiation on ficoll-isopaque separated cells against the antibody coated lymphocyte and erythrocyte targets. Background ^{51}Cr release as indicated by interrupted line. Period of incubation = 4 hours. Number of cells used - 10^6 per test making the effector to target cell ratios 20:1 for lymphocyte and 10:1 for erythrocyte target systems. Cell o——o is the same as E1 in Tables 5 and 6.

E. Discussion

Although normal individuals exhibit a wide range of variability in ECA, it appears possible to group them according to age and sex with some degree of predictability. Thus, post-climacteric individuals have significantly higher ECA than those in the prepubertal age group; prepubertal males have significantly lower ECA than post-pubertal males; preclimacteric females have significantly lower ECA than postclimacteric females; and females in their active sexual decades have significantly lower ECA than males of the same age. These findings suggest that male and female hormones may have some influence on the effector cells either qualitatively or quantitatively. It is of some interest to note that some of the females with low ECA were on oral contraceptive medication (B, B_1, B_2 and B_3 in Fig. 1) and that on one occasion when B was not on the "pill" her ECA became significantly higher than usual (see Table 2, Footnote c). These findings may be purely coincidental. On the other hand, they may reflect the effect of the oral contraceptives on ECA.

The low ECA of cell B cannot be attributed to inhibition by HLOC serum since its activity remains low in the absence of that serum (Table 4). This is supported by the finding that cell C (Fig. 1), which carries HLA-B 12, had a much higher ECA than cell B and yet had been previously shown to release more ^{51}Cr than cell B when both were compared as targets using HLOC serum. On the other hand, ECA is much lower in cell D which also carries HLA-B 12

(in addition to A 11). These findings, in contrast to others (81, 101) could possibly be explained on the basis of sex difference. In support of this are the data in Table 3; cell A not only consistently exhibited higher ECA than cell B against other targets, but also higher ECA than cells from the women whose sera were used. This argues strongly against the assumption that the low ECA in cell B and cell D is due to inhibition by HLOC serum.

It is not possible to define whether the diversity in ECA in this study is based on qualitative or quantitative differences of the effector cells. The data from the dose-response curves shown in Figure 2 would favor a qualitative difference, although it is possible that the proportion of effector cells is 30-fold greater in individual A than individual B. It is also possible that the age-related increase in ECA may be inversely related to the decreased proportion of T cells that occurs with age (76, 144, 154, 172).

The significance of variability of ECA in normal individuals is not know, but this study indicates that it is a factor that should be taken into account in future research in this system. In addition, measuring ECA using the lymphocyte target system may become clinically useful as another facet of immune function in disease states, or for monitoring patients under immunosuppressive therapy.

The first indication that different cell populations may be required to lyse different types of targets is the dissociation of

ECA's against two different targets as shown in Fig. 4. The lack of difference between ECA's of males and females against the erythrocyte target suggests that these cells, in contrast to the cells involved in the lymphocyte system, are not under the influence of sex hormones. In addition, these effector cells (for the erythrocyte target system) are not affected by immunosuppressive therapy whereas the cells involved in the lymphocyte target system are (data not shown).

By means of the adhering property to glass or plastic, of certain cells in our lymphocyte suspensions, a differential cytotoxic effect of the adherent and the non-adherent cells has been shown in the two ABCIL systems studied. The functionally radio-resistant adherent cell population with the morphology of macrophages or monocytes are exclusively cytotoxic to antibody coated erythrocytes. That they are not cytotoxic to antibody coated lymphocytes was supported by the fact that irradiation could abolish the cytotoxicity of Ficoll-isopaque separated cells against the lymphocyte target without any residual cytotoxicity which, if present, might have been contributed by the radioresistant cells. In addition, these adherent cells, known to carry Fc receptors, were able to block the cytotoxicity of the nonadherent cells, possibly by competing with the latter for the Fc of the antigen-complexed antibody on target lymphocytes.

On the other hand, the nonadherent cells with the morphology of small lymphocytes were the sole cytotoxic cells in the lymphocyte target system. It seems likely, therefore, that the adherent cells are the cells responsible for the lysis of antibody coated erythrocytes whereas the nonadherent cells are responsible for lysing non-erythroid target cells.

F. Conclusion

While sex difference in ECA against antibody-sensitized lymphocytes was seen in normal individuals in their active sexual age, this was not the case against antibody-sensitized eyrhtrocytes suggesting that different types of effector cells may be required for these two targets. Using the adherence property to glass or plastic, and functional radiosensitivity, it has been possible to identify two distinct populations of cells, each with a selective killing to only one of these two antibody-sensitized target systems. The adherent cells which are radioresistant in this system exhibit strong cytotoxicity against the antibody coated erythroid target while lacking any killing effect toward the antibody coated target lymphocytes. The nonadherent cells which are radiosensitive in this system, selectively kill the antibody coated lymphocyte target without possessing any cytotoxicity against the antibody coated target erythrocytes.

Addendum:

It had been suggested that the killer cells in this antibody dependent system be called K-cells (50). In a recent workshop report from the International Congress of Immunology held in Brighton, England, these killer cells have been classified further into K1, K2, B cells, macrophages and others (106). The non-adherent cells in this study are compatible with the K1 cells. It is not possible to define the adherent cells as the K2 or the macrophages at this time.

Since the completion of this study, another group of investigators (146) has also shown that different types of cells are required to lyse different antibody coated targets.

Chapter III. Specificity Studies in Antibody Mediated Cell Dependent Immune Lympholysis, with Regard to HLA-A, B & D

A. Introduction

Prior to the embarkation of the author on to the present study, McConnachie and Dossetor (109) in this laboratory had already been working on the ABCIL system using a modified technique of Canty and Wunderlich (22) who employed rocking motion in their cytotoxic assays. At that time, three humoral antibody systems namely, lymphocytotoxicity of Terasaki (CYT), leukoagglutination of Payne (AGG), and capillary agglutination of Thompson and Severson (CAT) had already been developed. Limited comparison of the ABCIL system with CYT suggested an increased sensitivity of the former in detecting evidence of tissue sensitization. Specificity of the reactions was partially defined by the HLA-A and B system (109).

At the beginning of the author's study, some modification of the technique was made. Experiments were done in test tubes and petri dishes concurrently and identical results were obtained. Since then, for convenience, all the experiments were carried out in test tubes.

It has been known since 1958 that pregnancy is a form of tissue sensitization (132, 185). It was ideal for this type of study because in the first place, the specificity of the sensitizing agent, as far as the A & B antigens of the HLA system is concerned,

in most cases is known; secondly, the number of immunizing HLA-A & B specificities is restricted (compared, for example, to multitransfused patients); and finally, the husbands are usually available for repeated or serial studies.

In this chapter, the author intends to show that although most ABCIL reactions could be explained on the basis of sharing of the A and B antigens of the HLA system directly or by cross-reactivity, many remained unexplained on that basis. Evidence will be presented to suggest that some of these latter reactions may be directed against the HLA-D locus antigens, as judged by differential lymphocyte and platelet absorption studies, with confirmation by MLC tests. Finally, a step-wise process will be formulated of how this test can be used to detect HLA-D locus antigens with selected sera.

For the sake of convenience, four separate but related sets of investigations will be presented in the following two sections. Section B 1-4 describes the principles of these groups of experiments. Section B 5 a-g describes laboratory methods. Section C 1-4 gives the four sets of data.

B. Materials and Methods.

1. Initial Study

Sera from 31 married women of varying parity were studied, both husbands and wives being bled at the time indicated in Table 7. These parous women were not selected at random from a large population so are not necessarily representative of all parous women. Sera were obtained from two control groups: women married for more than 1 year with no pregnancies and normal, presumably unsensitized, laboratory personnel. In nulligravid married women, sera were tested against husbands' lymphocytes; in the group of laboratory personnel, sera were tested against a five cell panel covering 16 of the known A and B locus antigens of the HLA system. In addition, selected women's sera were also reacted with panel cells that were HLA typed for antigens of the A & B loci to define their specificity. All sera were heat inactivated at 56° for 30 min. before use.

2. Correlative study using one non-HLA-A & B serum (STIN) against unrelated cells with respect to their ABCIL reactivity, MLC stimulatory activity and serum inhibitory effect in UMLC.

One unique serum from the initial study (serum from woman 20 in Table 7) was obtained from an Inuit (Eskimo) woman 10 years after her last (12th) pregnancy. As reported previously, this woman is HLA identical with her husband at the A & B loci but different at the HLA-D locus as evidenced from their capability to stimulate each other in MLC (47). As expected, this serum did not contain

any cytotoxin yet it had good ABCIL activity against her husband and some children (97). This serum was reacted with 33 unrelated individuals. Then a limited absorption study was carried out with platelets and lymphocytes to be described below. Lymphocytes from randomly selected individuals that were either ABCIL positive or ABCIL negative with this serum were then used in MLC tests with and without the addition of this serum.

3. Family study showing ABCIL detection of MLC (HLA-D) identity between HLA-A & B haploidentical individuals.

In the course of selecting a suitable kidney donor for a patient with chronic glomerulonephritis and end stage renal failure, a large family, with three generations, was used for family segregation studies. The segregation patterns of certain ABCIL sera were explored on the assumption that they might be directed against a specific HLA haplotype. This family will be designated as "Family F". Initially, 22 sera with known anti-HLA-A & B cytotoxic specificities were screened with the grandparents. From these, only two were suitable for subsequent testing with other members of this family in that each reacted positively with only one grandparent in ABCIL - a different grandparent for each serum. Serum 1 was a gift from N.I.H. (#1-04-9-05-08-01) and serum 2 was from woman #27 in Table 7. The specificity of both sera was anti-HLA-B 12 by lymphocytotoxicity, although sera 2 clearly reacted beyond HLA-A & B defined specifities in ABCIL against unrelated individuals (Table 9).

The results of ABCIL testing with these two sera against the family members in the second and third generation led to investigations in the MLC test involving all members of the family to be described below.

4. Studies in inbred Hutterites; "Tandem" ABCIL testing, using unabsorbed sera or sera that have been absorbed with lymphocytes from HLA-D homozygous individuals.

Hutterites are a group of anabaptist farming people who lead a closed communal life. They originated from the Austrian Tyrol almost 400 years ago whence they moved under the stimulus of persecution to Slovania, Hungary and finally Russia, where they lived for 100 years. A group emigrated to the United States in 1879. As a result of further persecution in the first world war, they decided to move again to Canada in 1918 and established three colonies in Southern Alberta (86). There are now close to 100 such colonies in this province.

Because of their high degree of inbreeding, they proved to be an ideal source for search of HLA-D homozygous cells. Such cells have become of great interest to transplantation laboratories over the world for their use as probes for identifying HLA-D antigens responsible for stimulation in MLC (49, 112, 178) and are believed to be more important than HLA-A & B in cross-matching for

transplantation (30, 57, 79, 167, 180, 184, 189).

More than 10 colonies (an average of 100 inhabitants per colony) have been studied to-date. Initially, individuals more than 8 years of age were HLA typed for antigens of the A & B loci. Then appropriate families were selected for further study in MLC. To-date, approximately 30 HLA-D homozygous cells with 10 different though undefined specificities have been found and are being characterized by Bo Dupont in New York and Rose Payne in Stanford.

Part of a large family is depicted in Figure 11. In this family Elias is homozygous for HLA-D though not for antigens at the A & B loci. His sister, Martha, is homozygous for antigens of all three loci, A, B, & D of the HLA. This homozygous D locus antigen has now been identified as DW2, which is the same as the LD-7a of Jersild and his associates (90).

The study to be further described involved the use of serum from Mary, who is married to Elias and has six children, against 32 unrelated cells in ABCIL. Then cells from these 32 individuals were used as responding cells to stimulation by cells from Martha in MLC.

5. Laboratory methods

a) Lymphocytotoxicity (CYT) of Terasaki (121).

Lymphocyte suspensions were prepared from heparinized blood and placed on a column of 0.2 mm diameter gum arabic coated glass beads contained in a nylon plugged plastic drinking straw for 3 min at 37°C to remove contaminating granulocytes and large mononuclear cells. The effluent lymphocytes were adjusted to 2×10^6 cells per ml in McCoy's medium with 30% FCS. One µl of this purified lymphocyte suspension was then placed in the wells of the disposable polystyrene microtitre trays (Falcon #3034) which contained 1 µl of test sera and covered with mineral oil to prevent evaporation. After mixing, the trays are incubated at 20° C for 30 min, then 5 µl of rabbit complement were added to each well, followed by another incubation period of 60 min before adding aqueous eosin (3 µl). Three minutes later 8 µl of formaldehyde was added, then the trays were covered with 50 x 75 mm. microscopic slides to flatten the oil droplets and read with an inverted phase contrast microscope using a 10 x objective. Living lymphocytes are small and refractile, and dead ones are larger and stained with eosin. A positive reaction is one with more than 50% cell death and a doubtful positive being one with increased cell death above controls but less than 50%.

b) Capillary agglutination (CAT) of Thompson and Severson (166)

This test uses crude leukocytes which were prepared from EDTA

blood by plasmagel sedimentation, contaminating erythrocytes being eliminated by hypotonic EDTA shock lysis and cellular debris removed by centrifugation through a column of 6% dextran. The leukocytes were then resuspended in gelatin, EDTA, phosphate saline buffer (GEPS) with 12.5% normal AB serum and adjusted to 20 - 30 x 10^6 cells per ml. Ten µl of this freshly prepared leukocyte suspension were mixed with 10 µl of test serum and incubated at 20° C for 10 min, then the mixture was drawn into microcapillary tubes which were sealed, centrifuged, and affixed to a metal plate at a 45° angle for 60 min with the cell pellets uppermost. In normal serum, the cells will stream at least 20 mm down the capillary tube. A streamer length of less than 50% of the normal was considered positive.

c) Leukoagglutination (AGG) of Payne (131), as modified by Schlaut.

This uses defibrinated blood with plasmagel sedimentation (10 ml blood:2.5 ml plasmagel or 4% polyvinylpyrolidone in saline). The supernatant was removed as leukocyte suspension and contains 2 - 6 x 10^6 leukocytes per ml. One drop (0.05 ml approximately) of this leukocyte suspension was added to one drop test serum in clean 10 x 75 mm glass test tubes, these were then gently mixed and incubated at 37° C for 75 - 90 min. After adding two drops of 6% acetic acid to lyse contaminating erythrocytes, the mixture was spread out in a thin layer on microscopic slides, allowed to dry,

and stained with Wright's stain. The dried slides were then covered with oil and examined microscopically. The reaction was considered positive if agglutination occurs.

d) Antibody mediated cell dependent immune lympholysis (ABCIL)

This test has been described in Chapter II. In the initial study, the effector and target cells were cultured in 1 ml with test serum present being 10%. In subsequent studies under headings 2, 3, and 4 the test was semi-miniaturized to a total volume of 0.25 ml with test serum present still being 10%. Such modification did not in any way interfere with the accuracy of the test. Since normal individuals' lymphocytes vary in their activity to kill in this system, only lymphocytes with good effector cell activity were used in all these studies.

e) One-way mixed lymphocyte culture tests (one-way MLC or UMLC) and serum inhibition in UMLC

This was done in triplicate according to a modification of the method by Bach (8). 10^5 responding cells, purified by the ficoll-isopaque separation technique, were cultured together with 10^5 mitomycin C treated stimulating cells for 5 days in 1 ml of medium 199 with 15% normal AB serum. Mitomycin C treatment consisted of exposure of the cells to 25 µg of mitomycin (8) for 30 min at 37° C followed by two washes to remove excess. Tritiated thymidine was added to the cultures 4 hours before they were processed for counting in a liquid scintillation (Packard) counter. The results were

expressed as stimulation indices (S.I.) calculated according to:

$$S.I. = \frac{R + Sm}{R + Rm} \quad \text{or} \quad \frac{\text{Cpm of responding cells + mitomycin C treated stimulating cells}}{\text{cpm of responding cells + mitomycin C treated responding cells}}$$

and relative response (R.R.) according to:

$$R.R.\ (\%) = \frac{\text{experimental cpm - background cpm}}{\text{average cpm of total response - background cpm}} \times 100$$

in which experimental cpm = cpm of a responding cell population to one specific stimulating cell; background cpm = R + Rm above; total response = sum of cpm of all the responding cell populations to the specific stimulating cell.

In experiments in which the inhibitory effect of serum was investigated, cultures of responding and stimulating cells as described above were made with and without the addition of the test serum to a final concentration of 1.5%. The enchancing or inhibitory effect was calculated from the following formula:

$$\%\ \text{change in S.I.} = \left(\frac{R + Sm + \text{serum}/R + Rm + \text{Serum}}{R + Sm/Rm + Rm} - 1\right) \times 100$$

with positive values indicating enhancement and negative values, inhibition by test serum.

f) Serum absorption. This was done by using the cell dosage of van Leewen and his associates (181). For absorption with

platelets, 1 ml of test serum was mixed with 10^9 platelets at 20° C with frequent shaking for 1 hour, twice. The absorbing dose for lymphocytes was much less, being 50×10^6 cells, twice, for 1 ml of serum.

g) Separation of T & B cells from PBL. This was done by following the method of Wisloff and Froland (195) using nylon wool. Two gm of nylon wool were packed into a 50 ml syringe wetted and warmed up to 37° C. Then PBL (70×10^6) prepared by the ficoll-isopaque separation technique was placed into the wool and incubated at 37° C for 45 min. The nonadherent cells were then flushed out in the effluent with warm medium 199 with 10% FCS.

These effluent cells were rich in SRBC rosetting cells compared with the unseparated PBL and were used as "T" cells.

With an 18 gauge needle connected to the syringe and immersed in cold medium 199 with 10% FCS, and with the plunger inserted, the adherent cells were eluted from the nylon wool into the cold medium by drawing the plunger back and forth. This yielded about 10% of the original PBL, was rich in non-rosetting cells with SRBC, and was used as "B" cells in experiment 3.

C. Results

1. Initial Study

Of the 10 married women with no prior pregnancies, only 1 gave a specific ^{51}Cr release of greater than 2% with the husband's PBL target. The mean ^{51}Cr release in this group of controls was -0.19 ± 1.40. Of the 60 ABCIL reactions by 12 normal sera against 5 PBL targets, there were 3 reactions with a ^{51}Cr release of greater than 2%, 2 greater than 3% and another 2, greater than 4%. The mean specific ^{51}Cr release from these 5 targets in the presence of sera and effector cells in this group of controls were 0.14 ± 0.25, 2.45 ± 1.12, 0.52 ± 0.83, -0.59 ± 0.93, and 0.07 ± 0.68% with the overall mean of the group 0.46 ± 1.26%.

In subsequent testing in the 31 married women with one or more pregnancies against their husbands (Table 7), all but the first 6 gave a specific ^{51}Cr release of >5%. Taking this as evidence of tissue sensitization at the 95% confidence level, 80% of these women would be considered to have been sensitized by their husbands. In those 28 instances where both ABCIL and CYT were done. CYT detected evidence of tissue sensitization in only 13 (46%) whereas ABCIL detected 22 (79%). Thus, ABCIL was able to detect presensitization in 33%, which would have been undetected by CYT alone. In the 15 CYT-negative sera in this series, 11 (73%) were positive by ABCIL.

Because the group under study was small and not randomly selected, the result does not permit any meaningful statistical analysis with

Table 7. ABCIL on 31 parous women against their husbands[a].

Woman	Gravida	Para	Period Postdelivery	Specific ^{51}Cr release (%)	CYT[b]
1	3	2	9 mo	-2, -1	-
2	4	3	3 mo	0, 1	-
3	4	4	4 wk	0, 0	+
4	1	1	2 mo	0, 1	-
5	3	3	9 mo	0, 1	-
6	2	2	6 wk	1, 1	+
7	3	3	2 mo	5, 5	-
8	1	1	9 mo	6, 6	-
9	5	5	6 mo	5, 8	-
10	2	2	12 mo	7, 7	-
11	3	3	2 mo	9, 9	-
12	4	4	5 mo	11, 13	+
13	4	3	5 wk	15, 15	-
14	4	4	11 mo	16, 18	-
15	3	3	9 mo	20, 20	-
16	3	2	10 wk	19, 21	N.D.
17	3	1	2 mo	20, 24	+
18	3	3	5 wk	22, 24	+
19	5	5	5 wk	28, 34	+
20	12	12	10 yr	32, 34	-
21	1	1	6 wk	33, 33	N.D.
22	5	4	3 mo	31, 39	+
23	3	3	8 wk	37, 39	+
24	3	3	3 mo	41, 45	+
25	5	4	7 wk	46, 46	+
26	3	3	10 wk	45, 47	N.D.
27	3	3	10 mo	47, 48	-

Table 7. (Continued)

Woman	Gravida	Para	Period Postdelivery	Specific ^{51}Cr release (%)	CYT[b]
28	3	3	3 wk	49, 50	+
29	3	3	11 wk	49, 51	+
30	4	4	7 mo	54, 57	-
31	4	4	10 wk	57, 58	+

[a] Ranked according to increasing percentage of specific ^{51}Cr release at 4 hours. Effector cells in each instance were from the wife.

b N.D. = not done.

regard to parity. The data do suggest, however, that ABCIL antibody may persist for longer periods of time after sensitization than CYT, as 9 women studied 6 months after parturition were CYT negative but ABCIL was positive in 7 (Table 8). In support of this suggestion was the finding that in 9 women, studied serially, there were 2 in whom both tests were positive at the time of delivery; ABCIL remained positive at 15 and 16 months, respectively by which time CYT was negative (Fig. 6).

Attempts to characterize specificity of the ABCIL antibody were made by reacting sera to test panel cells, only some of which shared wife-incompatible HLA antigens of the A and B loci of the husbands. Sera positive with each individual husband were not only positive with cells sharing husband's wife-incompatible HLA (A & B) antigens in all instances except two, but were also positive with cells sharing none of the husband's HLA (A & B) incompatible antigens; either directly or by cross reactivity as defined by Terasaki (120).

Of particular interest is the finding in family 1 that ABCIL was positive with each of 14 cells tested. In fact, the only instance that ABCIL was negative was when the woman's serum was reacted against her own cells (Table 9). These findings show that ABCIL reactivity in this serum was not restricted to HLA-A & B antigens, directly or by cross reactivity; whereas CYT, capillary agglutination, and leukoagglutination were strictly confined to A & B antigens of HLA.

Table 8. Results of CYT and ABCIL reactions with regard to after delivery to time of study (as indicated in Table 7).

Test results	CYT		ABCIL	
	+	-	+	-
< 6 months	13	6	18	4
> 6 months	0	9	7	2

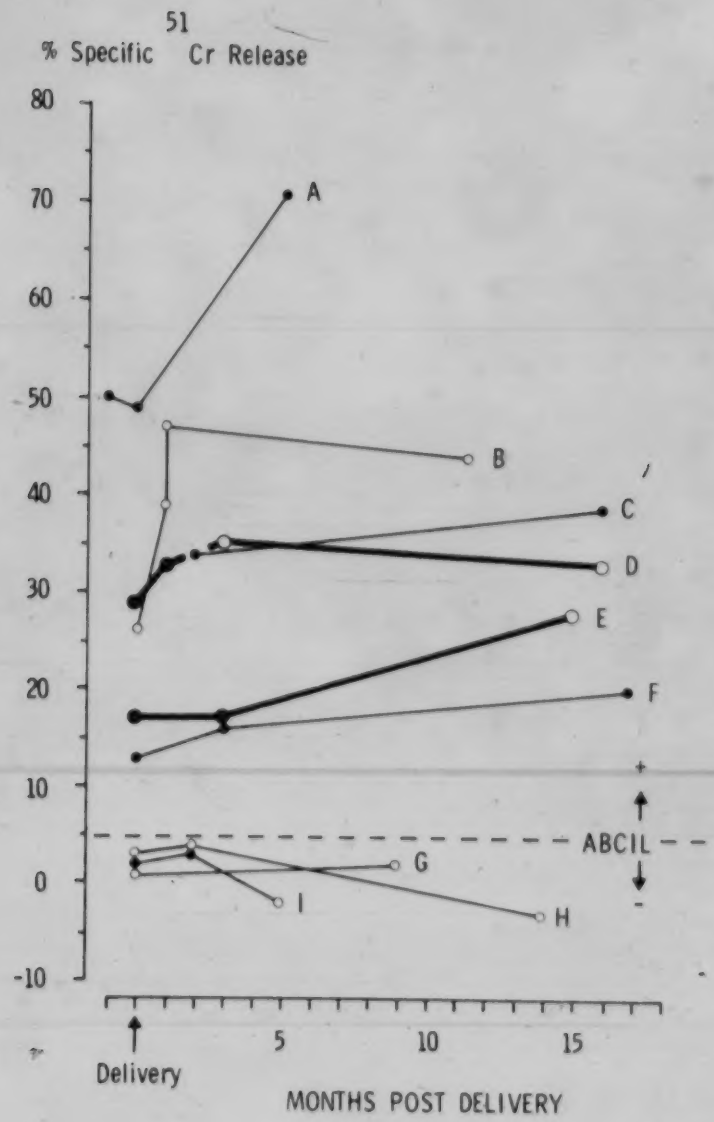


Figure 6. Serial study of CYT and ABCIL in nine couples. Couple D was the same as woman 27 in Table 7. •, CYT +; o, CYT -. ABCIL reactions, expressed as percentage of specific ^{51}Cr release, were positive in couples A, B, C, D, E, and F at various points studied, whereas CYT in couples D and E had become negative at 3 and 15 months postdelivery, respectively. Note also that in three ABCIL negative couples (G, H, and I), one was initially CYT positive (couple I).

Table 9. Correlative Study in Family 1[a]

HLA(A&B) Profile and Tests	WIFE	HUSBAND	No. of incompatible HLA(A&B) antigens													
			Direct													
			2	2	2	2	1	1	–	–	–	–	–	–	–	–
			Cross reactive													
			–	–	1	1	1	–	2	2	2	1	1	–	–	–
			Panel cells													
HLA(A&B) Profile:	A	2	3	→11	2	1	1	2	2	2	1	1	28	2	2	9	3
	A	3	→11	W32	→11	→11	→11		W30	W30	W30	10	W32	W29			9
	B	W35	→7	→12		→7	→7	→7	8	W22	8	W22	W22		5		
	B	14	→12	W35	→12	8	W15	27	→12	W40	W40	14	14	W40	W35	5	5
Tests: CYT			(+)–	(+)–	(+)–	–	–	–	(+)–	–	–	–	–	–	–	–	–
CAT			+	+	+	–	+	–	+	–	–	–	–	–	–	–	–
AGG			–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
ABCIL(%)[b]		-1	47	52	50	20	8	5	33	20	8	40	5	10	18	5	32
UMLC+(%)		16.6	91.6	88.0	83.3	7.6	78.6	86.1	62.5	79.4	75.3	86.2	69.2	74.4	83.7	78.6	ND[c]

[a] Arrow (→) indicates HLA(A&B) antigens similar to husband's; underlining, cross reactive with antibody to husband's wife-incompatible HLA(A&B) antigens. (+),previously positive.

[b] Expressed as percentage specific ^{51}Cr release at 4 hours.

[c] ND = not done.

In this and other families, there appears to be some similarity between ABCIL and antibody-induced inhibition of unidirectional mixed lymphocyte culture stimulation (Table 9). For this latter antibody Gatti et al (59) have also reported lack of restriction of antiserum specificities to the HLA antigens of the A & B loci.

Finally, when these results were analyzed quantitatively with regard to the number of husbands' wife-incompatible HLA-A & B antigens shared among test panel cells, directly or by virtue of known cross reactivity, the percentage of specific ^{51}Cr release cannot be correlated with the degree of HLA-A & B shared disparity (Fig. 7). This suggests that either ABCIL detects other histocompatibility antigens and not HLA-A & B or it detects other histocompatibility differences in addition to antigens of the A & B loci of the HLA system.

2. STIN serum study

Of the 33 cells reacted with STIN serum in the presence of good effector cells, 16 were positive (48%). Amongst both the ABCIL positive and ABCIL negative cells with the serum, 12 were selected so that 6 were from the negative group and the other 6, positive.

As shown in Table 10, cells A, B, C, D, E and F are ABCIL negative with STIN serum; cells G, H, I, J, K and L are positive. An absorption study was used to explore whether or not STIN serum was directed against a similar membrane bound determinant on different cells and whether or not this determinant is present on platelets.

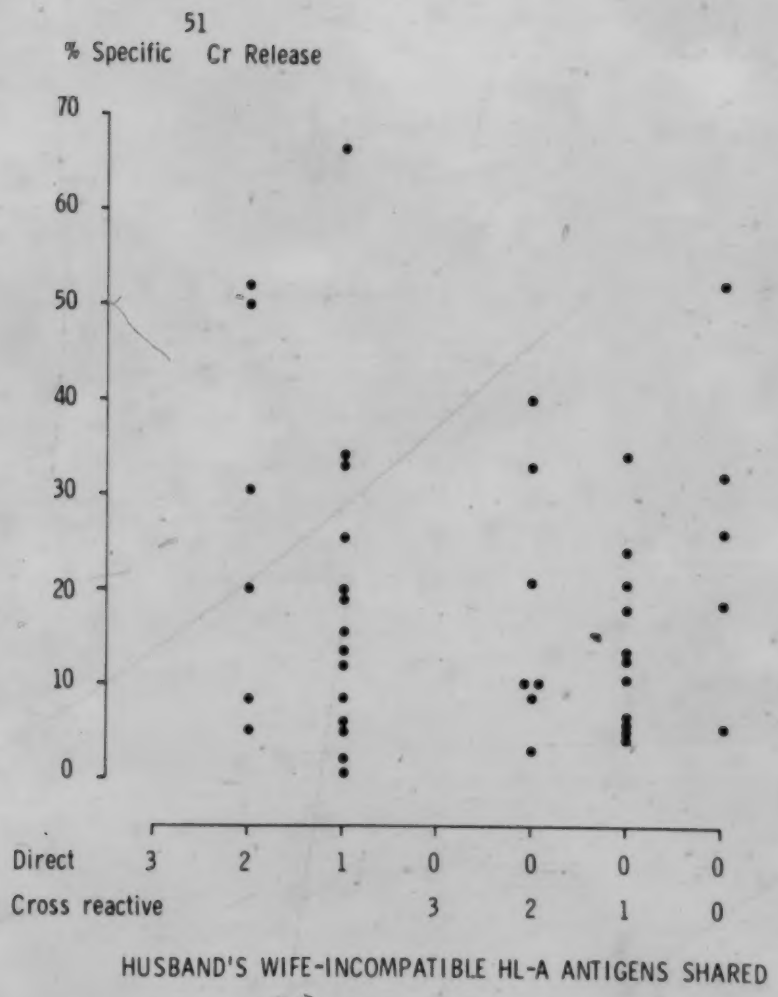


Figure 7. Quantitative analysis with regard to HLA-A and-B antigens shared with husbands, directly or by cross-reactivity, and the degree of target cell lysis as measured by percentage of specific ^{51}Cr release among unrelated subjects. No difference in percentage of specific ^{51}Cr release can be found among the groups that shared none or some HLA-A and-B similarities with the husbands.

Table 10. ABCIL reactions as measured by ^{51}Cr release, of 12 cells with race, sex and HLA(A&B) profile as shown, in the presence of STIN serum and effector cells.

Cells	Race[a]	Sex[b]	HLA(A&B)	Specific ^{51}Cr release[c](%)	
				Background	Experimental
A	C	F	A2,A11,B12,BW35	11.1	11.8
B	C	M	A3,AW31,BW17,BW40	7.6	10.1
C	C	F	A2,B7,B27	5.7	7.5
D	O	M	A2,B5,BW35	4.6	6.1
E	C	F	A11,AW32,B12,BW35	9.8	12.9
F	C	F	A1,AW31,B8,BW40	7.6	9.6
G	C	M	A1,A11,B7,BW15	6.7	40.8
H	C	F	A1,A11,B7,B8	7.2	33.1
I	C	F	A28,AW32,B14,BW22	4.4	18.7
J	C	F	A1,A2,BW15,BW21	11.5	26.8
K	O	M	A2,BW40	6.7	27.5
L	C	F	A27,AW32,B14,BW35	4.5	28.1

[a] C = Caucasoid, O = Oriental.

[b] F = Female, M = Male.

[c] after 3 hr incubation at 37° C in 5 % CO_2 atmosphere.

Lymphocytes and platelets from individual G were used at a dosage similar to that adopted by van Leeuwen et al (181). The absorbed sera were then used to react with cells G, H, I, J, and K. It is clear from this experiment that the ABCIL activity of STIN serum cannot be removed by platelets from G (Fig. 8). On the other hand G lymphocytes not only removes ABCIL activity against G but also activity against H, I, J, and K to a comparable extent. This suggests that ABCIL activity of STIN serum is monospecific and directed against a common determinant on G, H, I, J, and K lymphocytes, but not on their platelets.

The effects of STIN serum on one-way MLC are shown in Table 11. Blastogenic responses were strikingly different to stimulation by ABCIL negative as well as ABCIL positive cells, in the presence of STIN serum. With the exception of cells C and J, all other cells showed UMLC inhibition by STIN serum to stimulation by cells G, H, I, and J, all of which were ABCIL positive with STIN serum. These same cells were not inhibited as respondors in UMLC when stimulated by the ABCIL negative cells (to STIN serum) A, B, C, and D. On the contrary, many responses were enhanced, especially cells A, C, and J. Taking both enhancement and inhibition into consideration, it becomes reasonable to suppose that all the MLC responses to ABCIL positive cells were inhibited in the presence of STIN serum, although this inhibition might have been masked by an enhancing effect as in cells C and J. It is concluded from this experiment that ABCIL and UMLC inhibitory activities of STIN serum are highly correlated

Figure 8. Absorption Study. See text for interpretation.

Table 11. Effect of STIN serum on MLC involving cells that were ABCIL negative (A-D) and positive (G-J)

% change in S.I. by STIN serum [a]

Responding cells	Stimulating cells (Mitomycin treated)								Mean ± SEM	Mean Difference	P
	Am	Bm	Cm	Dm	Gm	Hm	Im	Jm			
A		+107	+147	+155	-58	-67	-32	-50	+136 ± 15 -52 ± 7	188	< 0.0005
B	+28		-36	-44	-42	-51	0	-52	-17 ± 23 -36 ± 12	19	N.S.
C	+129	+257		+214	+20	-38	+9	+71	+200 ± 37 +15 ± 22	185	< 0.005
D	+1	+41	-7		-55	-71	-49	-39	+12 ± 15 -53 ± 7	65	< 0.01
G	+1	+51	+43	0		-58	-61	-60	+24 ± 13 -60 ± 1	84	< 0.001
H	-29	-6	-20	+2	-61		-78	-69	-13 ± 7 -69 ± 5	56	< 0.001
I	+67	+40	+59	+16	-52	-69		-68	+45 ± 11 -63 ± 5	108	< 0.0005
J	+188	+202	+168	+224	+27	+9	-46		+195 ± 12 -3 ± 22	198	< 0.0005

[a] As compared with cultures in the abscence of STIN serum. Positive values indicate enhancement, negative values, inhibition, by STIN serum.

and that the inhibitory activity of this serum is exerted on the stimulating cells in MLC.

This is in contrast to others' experience that anti-HLA (? A & B) antibodies inhibit both the stimulating and responding cells (27, 59, 142, 168). In view of the recent findings that B-cells are the stimulating cells in MLC (29, 139, 140, 193) (and experience in this laboratory is in agreement with this belief), B-cells and T-cells from individual G were prepared from peripheral blood lymphocytes by the nylon wool separation technique and used as targets in ABCIL with two sera, one (IMAH) with anti-HLA-B7 specificity (entirely absorbable with platelets from individuals with HLA-B7), the other being STIN serum. As can be seen in Table 12, IMAH serum reacted with all three targets from individual G, whereas STIN failed to react with the T-cell target, suggesting that STIN serum was detecting an antigenic determinant present only on the B-subpopulation of lymphocytes.

The final experiment in this study was based on the above findings. Since a) ABCIL activity of STIN serum appeared to be directed against a common determinant on B-lymphocytes of ABCIL positive individuals but not on their platelets, and probably not T-lymphocytes, and b) this same serum could inhibit the MLC reactions by acting on the stimulating cells (presumably B-cells) again from ABCIL positive individuals, it was conceived that ABCIL activity of STIN serum could be directed against a MLC gene product or lymphocyte

Table 12. ^{51}Cr release from unseparated PBL, B-cells and T-cells from individual "G" in the presence of IMAH (anti HLA-B7) and STIN (? anti HLA-D) serum and effector cells.

Serum	% ^{51}Cr release[a]		
	PBL	"B"	"T"
(Background)	5.0	7.4	3.9
IMAH	35.0	31.1	19.3
1:10	20.9	17.3	9.1
STIN	29.7	31.0	5.7
1:10	16.7	18.6	5.3
% SRBC rosette[b]	36	1	50

[a] after 3 hr incubation at 37° C, 5 % CO_2

[b] see reference 18

activating determinant (Lad) present on the cell surface of B-lymphocytes. Furthermore, it was reasonable to assume that ABCIL positive individuals could have a common Lad. If this were true, then ABCIL positive cells responding to other ABCIL positive cells in MLC should give a stimulation index lower than if they were responding to cells that were ABCIL negative with STIN serum or lacking the same common Lad. This assumption was tested by employing cells G, H, I, J, K, and L in MLC, in response to stimulation by these same cells, and by 6 ABCIL negative cells, A, B, C, D, E, and F. As shown in Table 13, the mean S. I. of each of these ABCIL positive cells responding to other ABCIL positive cells was lower than the mean of their S.I. to ABCIL negative cells, and in 4 individual instances the lower S.I. was significant. This finding strongly supports the assumption that the ABCIL activity of STIN serum is directed against Lad on lymphocytes.

3. Studies in family F

The HLA profiles of the A & B locus antigens of all the members of this family and the ^{51}Cr release using serum 1 and serum 2 in ABCIL are indicated in Table 14. As judged by the complement dependent lymphocytotoxicity test both sera had antibody against HLA-B12. However, there was no HLA-B 12 in this family, and these ABCIL reactions could not be explained in terms of their known HLA-A & B reactivity. Serum 1 was ABCIL negative with cells of the grandfather but positive with the grandmother. The converse was true with serum 2. If we assign a and b as haplotypes A2, BW15

Table 13. MLC reactions, expressed as stimulation indeces (S.I.) of 6 ABCIL + cells responding to themselves and to 6 ABCIL - cells by STIN serum.

Responding cells	S.I. Stimulating cells (Mitomycin treated)												mean ± SEM	mean difference	% lower	P
	Am	Bm	Cm	Dm	Em	Fm	Gm	Hm	Im	Jm	Km	Lm				
G	25	27	30	23	26	12							23.8 ± 2.5	7.4	31.1	< 0.05
								17	11	11	17	26	16.4 ± 2.7			
H	22	24	18	11	15	15							17.5 ± 2.0	1.9	10.8	N.S.
							11		8	16	20	23	15.6 ± 2.7			
I	56	74	73	63	84	71							70.2 ± 3.9	22.6	32.2	< 0.005
							50	41		44	42	61	47.6 ± 3.7			
J	22	27	17	21	25	9							20.2 ± 2.6	12.0	59.4	< 0.005
							5	10	7		9	10	8.2 ± 1.0			
K	15	23	16	22	23	24							20.5 ± 1.6	11.7	57.1	< 0.0005
							8	12	8	7		9	8.8 ± 0.8			
L	59	26	44	28	54	36							41.2 ± 5.5	11.8	28.6	N.S.
							41	30	13	33	30		29.4 ± 4.5			
mean ± SEM	32.2 ± 3.4						21.0 ± 2.8							11.2	34.8	< 0.01

Table 14. ABCIL reactions of serum 1[a] and 2[b] in family "F"

Targets	HLA(A&B) haplotypes		% ^{51}Cr release		
			Background	Serum 1	Serum 2
Father	A2,BW15;A2,B5	ab	3.1	5.2	19.7
Mother	A1,BW17;A2,BW35	cd	2.9	32.0	4.4
Eleanor	A2,BW15;A1,BW17	ac	4.3	20.0	20.9
Don	A2,BW15;A1,BW17	ac	7.0	21.4	19.7
Janet	A2,BW15;A2,BW35	ad	4.5	6.3	26.3
Terry	A2,BW15;A2,BW35	ad	5.1	7.2	23.4
Gordon	A2,B5,A1,BW17	bc	5.7	20.9	9.5
Norman	A2,B5,A1,BW17	bc	3.9	17.5	4.8
Ruth	A3,BW35;AW32,B14	xy	4.1	17.6	6.8
Brian	A2,BW15;A3,BW35	ax	5.2	30.8	28.1
Laurie	A2,BW15;A3,BW35	ax	4.2	31.5	29.7
Bruce	A2,BW35;AW32,B14	dy	5.4	6.1	11.1

[a] Serum 1 = NIH (#1-04-9-05-08-01) with anti HLA-B12 specificity by cytotoxicity.

[b] Serum 2 = # 27 in Table 7, also with anti HLA-B12 specificity by cytotoxicity.

and A2, B5 of the grandfather; c and d as haplotypes A1, BW17 and A2, BW35 of the grandmother; and x and y as haplotypes A3, BW35 and AW32, B14 of the daughter-in-law, Ruth, then the positive ABCIL reactions of serum 1 could be shown to segregate with haplotype c (and haplotype x) (Figure 9); and positive ABCIL reactions of serum 2, with haplotype a. However, serum 2 was also weakly positive with cells from Bruce who carried haplotype d rather than haplotype a. But this reaction was equivocal.

Thus, in this family, these two sera contained ABCIL reactivities which segregated with two distinct HLA-A & B haplotypes, a and c, one from each grandparent, and yet the complement dependent cytotoxic specificities were unrelated to the A & B locus antigens of these haplotypes. These two haplotyes were inherited by Eleanor and Don (Figure 9).

By chance, each of these two ABCIL reactive sera also differentially marked Terry and Ruth, two parents in the second generation, although serum 1 was now marking haplotype x from outside the family and not haplotype c. Haplotypes a and x were inherited by Brian and Laurie in the third generation.

The next step was to investigate whether the determinants on lymphocyte targets as detected by serum 1 in this family was of the same specificity or was different. This was carried out by absorption studies with lymphocytes and platelets from Eleanor, Laurie and Terry (Figure 10). The results showed that ABCIL

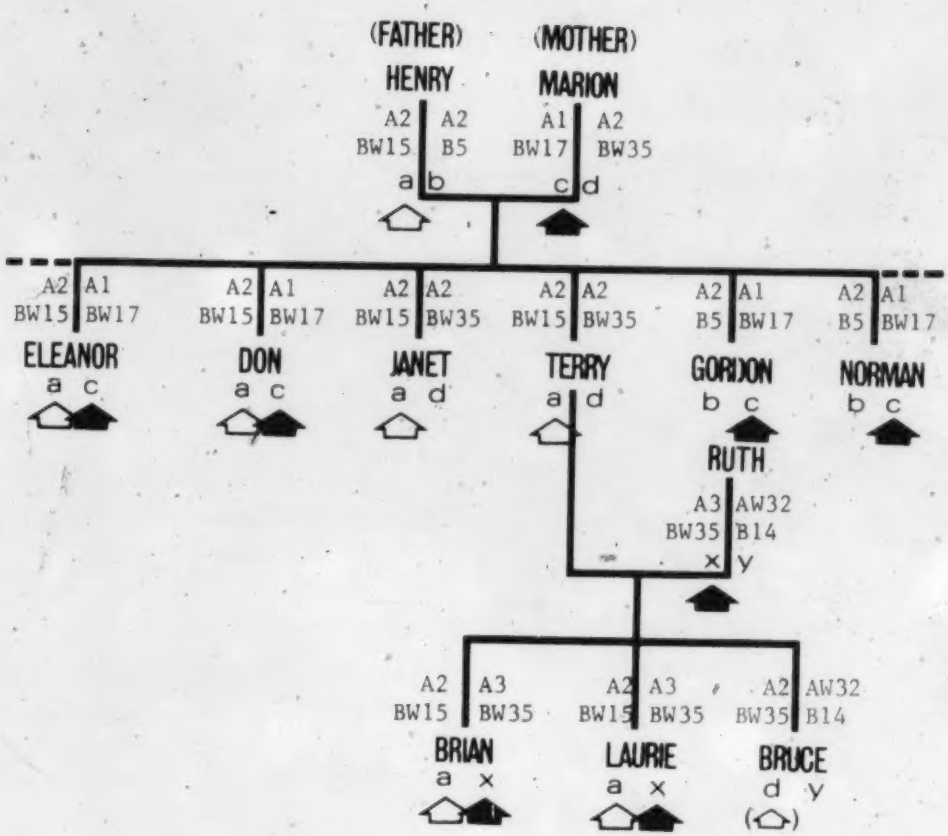


Figure 9. ABCIL segregation of sera 1 and 2 in family "F". Positive ABCIL reactions with serum 1 indicated by solid arrows and with serum 2, open arrows. (Other members in the second generation are not shown in this diagram).

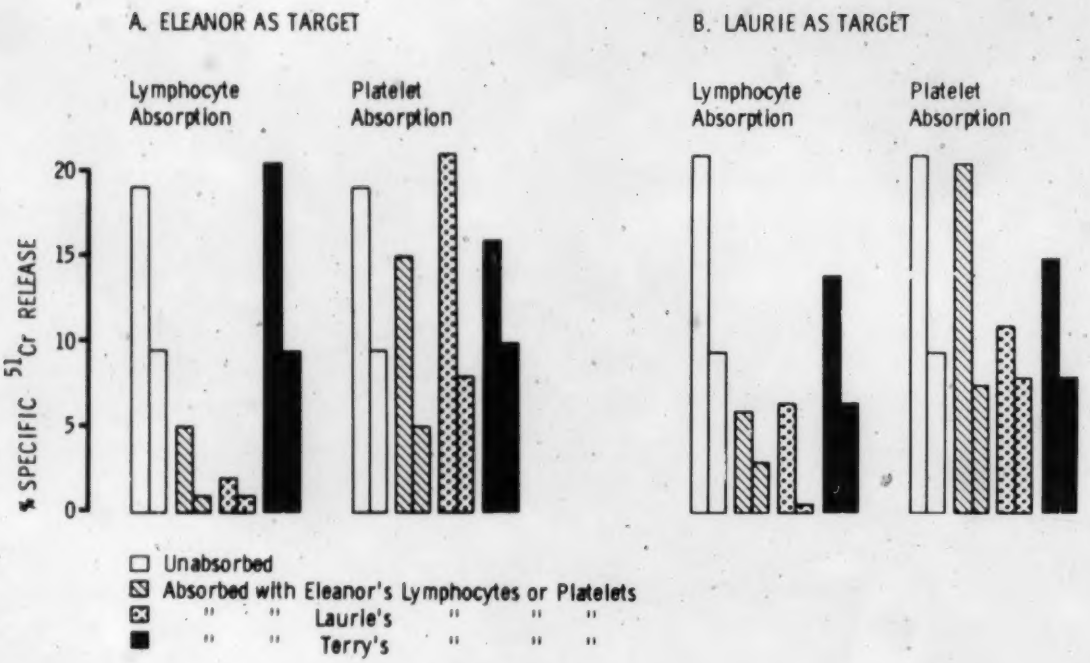


Figure 10. ABCIL absorption study. Serum 1 diluted 1:4 absorbed with lymphocytes (10×10^6 per 0.2 ml) and platelets (0.2×10^9 per 0.2 ml) from Eleanor, Laurie and Terry for one hour, twice at room temperature, then reacted in ABCIL with Eleanor and Laurie at 1:4 and 1:20 dilutions. Results expressed as activity left after absorption in percentage of specific ^{51}Cr release.

reactivity of the serum against haplotype c (Eleanor) could be removed by lymphocytes bearing haplotype x (Laurie), and vice versa. Terry's cells were used as controls in these absorption studies and did not remove ABCIL activity from serum 1. Results with platelet absorption were less conclusive but suggested that ABCIL activity could not be absorbed by platelets at the dosage used. On the basis of these differential absorption studies, it was believed that a factor coded for by a gene linked to haplotype c might be identical to the factor coded for by a gene linked to haplotype x (on the HLA chromosome) and that both were expressed on the lymphocyte membrane.

Hence, in this family, two HLA-A & B identical sib pairs, Eleanor and Don in the second generation and Brian and Laurie in the third generation, had lymphoid cells that behaved identically by the ABCIL test with these two selected sera, both directly and by absorption. These two HLA-A & B identical sib pairs had one HLA-A & B haplotype in common: A2 and BW15, derived from the grandfather, and marked by serum 2. The other A & B haplotypes, however, were different and were derived from unrelated sources.

Since genes of the D locus are closely linked to genes of the A and B loci on the HLA chromosomal region and code for lymphocyte activating determinants responsible for stimulation in MLC, the next step was to test whether the ABCIL reactivity of serum 1 was directed towards such determinants on lymphocytes of Eleanor (haplotype c) and Laurie and Brian (haplotype x). If

this were the case, then Eleanor's lymphocytes should not stimulate lymphocytes from Laurie and Brian, and vice versa. This was accomplished by performing MLC studies within this family. In three separate experiments, lymphocytes from Laurie consistently failed to stimulate or respond to lymphocytes from Eleanor, although Laurie's cells were capable of a full response to and stimulation by cells from her parents and other related or unrelated persons except Brian who was HLA identical to her at the A & B loci. Brian's cells, on the other hand, failed to respond to Eleanor's but on one occasion did stimulate hers to a stimulation index (S.I.) of 6.5, although this figure might still represent non-stimulation when compared with other S.I. of Eleanor's cells responding to those of other individuals.

The data of the 2nd of three experiments are shown in Table 15 and were analyzed for the stimulation of haplotype c on x, and vice versa, with other members of this family (Table 16). These data showed that cells which differed by one serologically defined HLA haplotype (A & B) had a mean S.I. of 28.6 $\pm$ 14.9, unless this serologically defined difference was due only to haplotype x instead of c (or vice versa), where the mean S.I. was 3.0 $\pm$ 2.3. Similarly when cells differed by two HLA-A & B haplotypes, the mean S.I. was 38.0 $\pm$ 18.4 unless one of these two A & B haplotype differences was due to haplotype x instead of c (or vice versa), where the mean S.I. was 21.1 $\pm$ 15.4. The latter was consistent for a single HLA-D

Table 15. Mixed lymphocyte culture tests done within family "F" plus an unrelated individual (expt.2). Results given as counts per min. Stimulation indeces given in parenthesis.

Responding cells	Stimulating cells								
	Eleanor (ac)	Terry (ad)	Norman (bc)	Gordon (bc)	Bruce (dy)	Laurie (ax)	Brian (ax)	Ruth (xy)	Unrelated
Eleanor (ac)	204	6529 (32)	6871 (33.7)	9461 (46.4)	12216 (59.9)	399 (1.95)	1323 (6.5)	9592 (47)	13452 (65.9)
Terry (ad)	8505 (49.7)	171	7884 (46.1)	9614 (36.2)	4572 (26.7)	9517 (55.7)	8576 (50.2)	15308 (89.5)	15273 (89.3)
Norman (bc)	5381 (24.9)	8974 (41.5)	216	251 (1:2)	6342 (29.4)	3102 (14.4)	6613 (30.6)	11171 (51.7)	11054 (51.2)
Gordon (bc)	7232 (25.8)	8145 (29.1)	201 (0.7)	280	9990 (35.7)	4306 (15.4)	6387 (22.8)	10912 (38.9)	6673 (23.8)
Bruce (dy)	11624 (38.4)	3245 (10.7)	9176 (30.3)	12256 (40.4)	303	11407 (37.6)	11976 (39.5)	9722 (32.1)	12612 (41.6)
Laurie (ax)	1132 (2.3)	5532 (11.3)	3734 (7.4)	3644 (7.4)	10756 (21.9)	491	381 (0.8)	9889 (20.1)	7683 (15.6)
Brian (ax)	3444 (1.4)	10683 (4.4)	12689 (5.2)	9348 (3.9)	18046 (7.4)	1187 (0.5)	2423	17897 (7.4)	19466 (8.0)
Ruth (xy)	13079 (18.5)	16042 (22.6)	9627 (13.6)	12986 (18.3)	16756 (23.7)	21481 (30.3)	21608 (30.5)	708	19729 (27.8)
Unrelated	10278 (32.6)	10510 (33.4)	9627 (30.5)	11124 (35.3)	9682 (30.7)	10704 (34.0)	14910 (47.3)	13384 (42.5)	315

Table 16. Mean stimulation indeces in one-way MLC, as condensed from Table 15 and group into 5 categories: 0 haplotype difference by both HLA-A&B and HLA-D, one haplotype difference by -A&B but 0 by -D, one haplotype difference by both -A&B and -D, two haplotype difference by -A&B but one by -D, and two haplotype difference by both -A&B and -D.

HLA-A&B defined haplotype difference	0	1		2	
ABCIL defined HLA-D haplotype difference	0	0	1*	1*	2
number	(4)	(4)	(18)	(14)	(32)
mean	0.8	3.0	28.6	21.1	38.0
S.D.	0.3	2.3	14.9	15.4	18.4
		N.S.		N.S.	$p < 0.005$
pooled *			(32)		
mean			25.3		
S.D.			15.4		
				$p < 0.05$	

haplotype difference.

This finding was confirmed in the third experiment in which the cells of 12 members of this family and an unrelated individual were reacted in MLC giving 137 combinations, each tested in triplicate and all done at one time. Cells that differed by two HLA-A & B haplotypes gave a mean S.I. of 31.2 $\pm$ 28.3 (N=47), except for the group where one of these two haplotype differences was due to haplotype x instead of c (or vice versa) where the mean S.I. was 15.0 $\pm$ 12.5 (N=24). This difference is highly significant ($p<.005$). There is no significant difference between this mean S. I. of 15.0 $\pm$ 12.5 and the mean S.I. of 20.4 $\pm$ 18.1 (N=52) for cells differing by one haplotype (exclusive of single differences due to haplotype c or x). When data from these latter two groups are pooled, the pooled S.I. is 18.7 $\pm$ 16.6 (N=76). This pooled S.I. is significantly different ($P<.01$) from the mean S.I. of 31.2 $\pm$ 28.3 for cells that differ by two haplotypes exclusive of x and c differences. Thus, experiment 3 confirms the results of the 72 MLC combinations of experiment 2. It was concluded from these studies that cells with haplotype c and x were identical in their capacity to stimulate or respond in MLC.

Thus, serum 1 in these two families marks a common MLC (HLA-D) gene product on lymphocytes, though this is associated with HLA-A1 and BW17 in family F, and A3 and BW35 in Ruth's family. It is reasonable to assume also that ABCIL reactions of serum 2 marks an

MLC gene product segregating with HLA-A2 and BW15, although this study only shows that it is not marking those A & B locus antigens detected by this serum in complement dependent lymphocytotoxicity reactions.

4. Identification of HLA-D locus antigens DW2 in unrelated individuals using "tandem" ABCIL testing before and after serum absorption with cells homozygous for DW2.

The MLC studies in 3 related Hutterite families in Figure 11 are as follows: In the family to the right of the figure, the mother is HLA homozygous for A10, BW16. However, when her cells were used as stimulating cells in MLC against her childrens', blastogenic response occurred in every instance; therefore, this A & B locus homozygous cell was not homozygous for HLA-D. Both parents of the family in the middle of the figure are homozygous for antigens of the A & B loci; the father for A3, BW18 and the mother for A3, B7. When these two cells were tested against their children, it became clear that the mother was also homozygous for the D locus antigen but the father was not. In the family to the left of the figure, both parents have the A3, BW18 haplotype and their daughter, Martha is homozygous for A3, BW18. In addition, she is also homozygous for the HLA-D locus antigen which has been identified as DW2. Furthermore, DW2 was also present on the A10, BW16 haplotype of the mother. Thus, the mother, Elias and the three youngest daughters of this family are homozygous for DW2.

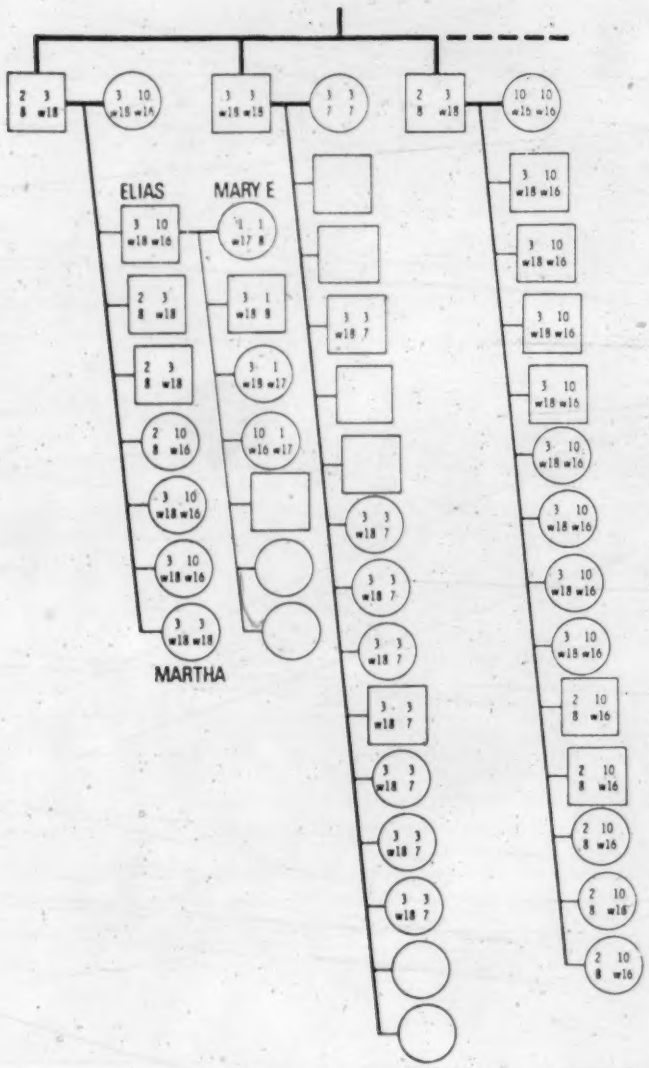
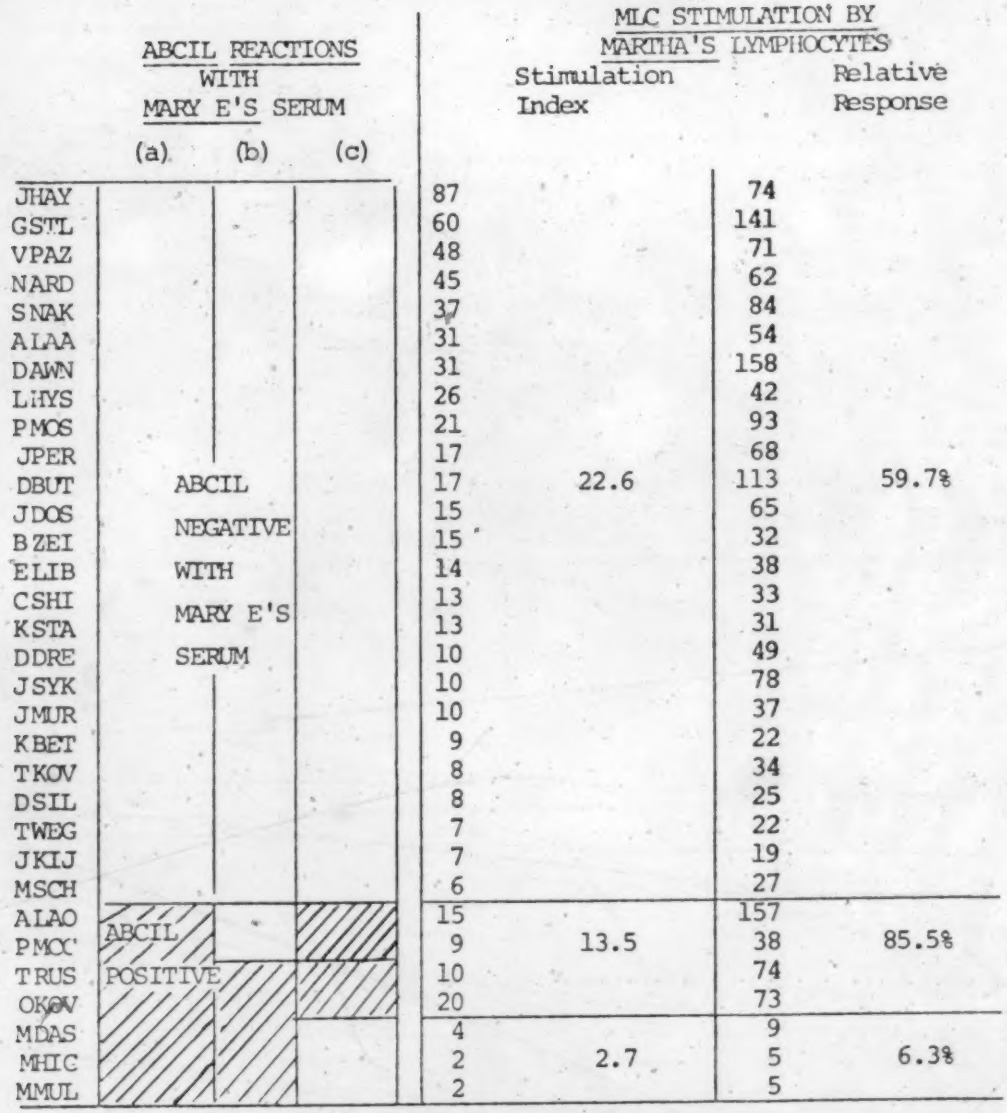


Figure 11. Three related families in a Hutterite colony. See text for detailed discussion.

This was judged by the non-stimulation among them in one-way MLC; yet when stimulated by other members of the family they responded normally.

The finding that Elias is homozygous for HLA-D is most interesting because his wife, Mary E, has 6 children and conceivably might have ABCIL antibody against DW2. Her serum was, therefore, tested with her husband, Martha and 32 other unrelated non-Hutterite cells. Absorption studies indicate that her serum contained anti A10 (as it was positive with all the A10 cells tested) which could be removed by her husbands' platelets; in addition, anti DW2 was also present and could be removed by her husband's as well as Martha's lymphocytes.

Figure 12 shows the reactivity of the ABCIL antibody in this serum against the 32 unrelated non-Hutterite cells of which seven were positive with the unabsorbed serum. After absorption with Elias's platelets cells ALAO and PMCC, both carrying HLA-A10, became ABCIL negative, though they remained positive with her serum which had been absorbed with Martha's lymphocytes. On the other hand, absorption of Mary E's serum with Martha's lymphocytes removed ABCIL reactivity against cells MDAS, MHIG and MMUL which now became negative, though they were still positive with the husband platelet absorbed serum. These findings suggest that cells MDAS, MHIG and MMUL might be carrying DW2 that Elias and Martha carry and this can be tested in MLC.



(a) Unabsorbed serum
(b) Serum absorbed with husband's platelets
(c) Serum absorbed with Martha's lymphocytes

Figure 12. Correlation of ABCIL reactivity with Mary E's serum and MLC response to Martha's lymphocytes among 32 non-Hutterite cells.

In this same figure the MLC responses of these 32 cells to stimulation by cells from Martha are also shown. It can be seen that the three cells positive by ABCIL with the unabsorbed Mary E serum but negative with Mary E serum after absorption by Martha lymphocytes all had low stimulation indices with a mean of 2.7 and a low relative response with a mean of 6.3. These are compatible with non-stimulation by Martha's lymphocytes and indicates that one of the two HLA-D antigens of cells MDAS, MHIG and MMUL is DW2.

D. Discussion

In the initial study the 46% overall positive results by CYT in this small group of women reacted against their husbands is in agreement with larger series (163). Evidence is accumulating, however, which shows that CYT alone is by no means adequate for detecting presensitization. At least another one-third would have been missed if ABCIL was not performed. In 505 consecutive women studied at the time of delivery in this laboratory, against a panel of five cells, CYT detected only about 60% of all of the positive sera. The other 40% of reacting sera were detected by the capillary agglutination test of Thompson and Severson (166) or by leukoagglutination (46). This clearly shows that CYT alone is not adequate for the purpose of detecting histoincompatible antigenic sensitization and brings up the question of how reliable is the concept of the so-called "nonresponders" who do not produce cytotoxic antibody after antigenic exposure (128). This question is particularly relevant when it comes to studying patients (see Chapter IV).

ABCIL can detect presensitization in more than one-half of the CYT-negative women. This is not attributable to differences in sensitivity of the two tests. ABCIL reactivity, although more sensitive than CYT, was negative in two of the women who were CYT-negative. Thus, increased sensitivity of CYT is not the explanation for the longer duration of ABCIL antibody reactivity in certain parous women's sera with time after the last delivery. Earlier

experience in this laboratory also suggests that they are different antibodies (97). It is not understood why some parous women produce one and not the other type of antibodies, but it is pertinent that doing any single test, no matter how sensitive it may be, is insufficient for the detection of all humoral evidence of tissue sensitization.

The problem of specificity in the initial study is disturbing, although now, by doing more and more differential platelet and lymphocyte absorption studies, the author finds that most ABCIL positive parous sera have specificities directed against the A and B locus antigens of the HLA system. But it was encouraging to find that a serum so widely reactive against unrelated individuals could be shown to segregate with a haplotype of the HLA-A & B and presumably HLA-D loci, in an individual family. This finding, together with findings in the final study, indicates that the ABCIL technique, using appropriate serum before and after removal of an HLA-D specificity by absorption can be used to identify that particular antigen in unrelated individuals, disregarding how broadly specific that serum may be.

In the STIN serum study, although it appears convincing that this serum could be detecting a common HLA-D antigen in those ABCIL positive individuals, the proportion of cells so detected was high. More cells are being tested at this point in order that a

realistic gene frequency of the antigen being detected can be estimated in the general population. One other intriguing finding is the rather constant degree of ^{51}Cr release from the "B" cell target compared with that from the unseparated PBL. One would expect a much higher degree of ^{51}Cr release from the former target if indeed this serum was reacting only with B-cells. Although this apparent discrepancy remains unexplained it is nevertheless clear that this serum did not react with the T-cells and that agrees with the results of UMLC inhibition by this serum.

The engagement of this laboratory in the search for HLA-D homozygous cells, or MLC typing cells, in the Hutterites has turned out to be very rewarding. This inbred population has so far contributed no less than 30 HLA-D homozygous cells of 10 different specificities. Such cells are not only useful as MLC typing cells, they are also ideal for absorbing out one particular HLA-D specificity from serum for HLA-D serology using the "tandem" ABCIL technique. In addition, because of their 'grand' multiparity, these Hutterite women are not infrequently found to have good ABCIL antibodies in their serum, often many years after their last pregnancy by which time any complement dependent type of cytotoxic antibody would long have disappeared. For some obscure reason, the antibodies in such sera are usually non-HLA-A & B and cannot be removed by platelets. It is quite possible that many of these sera may contain antibodies that are HLA-D specific and they are under

intensive study at present. It should be pointed out that such non-HLA-A & B antibodies usually give rise to only a low degree of ^{51}Cr release from positive PBL targets, in contrast to HLA A & B antibodies, although by serum dilution studies they appear to be strong. This finding is very suggestive of such antibodies reacting with only a subpopulation of cells in the PBL such as B-cells. To clarify this point, cells from patients with chronic lymphatic leukemia (CLL) are being used as targets for these sera. An increase of ^{51}Cr release from these targets that are positive would indicate that these sera react only with B-cells, since CLL cells are almost entirely B-cells (31, 130).

At the time of this writing, van Rood and his co-workers have reported the use of another serological method to detect these HLA-D antigens called the long, B-cell lymphocytotoxicity test (190). They also found that these HLA-D antigens (MLC determinants) are present only on B-cells, therefore they enriched B-cells from PBL and used them in the conventional complement dependent lymphocytotoxicity test with one modification - prolonged incubation time. Their results were very encouraging in that two sera after pooled platelet absorption to remove anti-HLA-A & B specificities marked cells that carry a known MLC factor in complete concordance with the results obtained by using MLC typing cells.

At present long B-cell cytotoxicity testing (B-CYT) is also under study in this laboratory. Preliminary results fail to show

a correlation of certain sera behaving in B-CYT and ABCIL. Of special interest is the STIN serum, though very reactive in ABCIL this serum was completely inactive in B-CYT. Moreover, this serum has been tested by Walford in Los Angeles with 70 CLL cells in cytotoxicity with uniformly negative results. This has once again confirmed our earlier finding that ABCIL antibody is different from the complement dependent antibody and suggests that both techniques may be equally useful in serologic typing for Ia-like and HLA-D specificities as well, perhaps, as membrane antigens that do not segregate with HLA.

E. Conclusion

The ABCIL technique has been used in four separate but related studies to investigate the specificities of certain multiparous human sera within the HLA system. Through studies with differential lymphocyte and platelet absorption, family segregation and by employing the MLC test and serum inhibition in one-way MLC, convincing evidence has been obtained which indicates that certain selected sera may contain anti-HLA specificities directed against the D locus antigens or MLC determinants. It seems reasonable to conclude that this technique may be useful as a serological means for MLC typing.

Chapter IV. The significance of ABCIL antibody in kidney Transplantation

A. Introduction

Prior to 1960, patients with chronic renal failure invariably died from lack of effective therapy other than conservation, that is: maintenance of fluid and electrolyte balance, limited protein intake, control of blood pressure, treatment of superimposed infections, plus other palliative and supportive measures. On a regime like this, patients could survive from several months to several years depending on the cause of their renal failure, but the quality of life was often close to intolerable.

The major cause of chronic renal failure is glomerulonephritis, an inflammatory group of diseases of diverse origins affecting the filtering system of the kidney, resulting in retention of toxic metabolic products and, with time, shrinkage of the kidneys to the point of non-function eventually; and glomerulonephritis strikes, predominantly, young men and women in their productive periods of life. So, every year, young men and women died in chronic renal failure.

Then, around 1960, two major developments took place which, for the first time in medical history, offerred two effective ways of treating these chronic renal failure patients, each complementing the other. Today, 15 years later, young men and women still die of chronic renal failure, but many have been saved and

rehabilitated. These two forms of treatment are hemodialysis (150) and kidney transplantation (78, 115). Both forms of therapy, in fact, existed much earlier. The former, for example, had been used for treating patients with acute, reversible renal failure (113). But it was not until the late 1950's that repeated access to the blood stream was made possible for long-term use without the necessity of vascular cannulation each time for each dialysis (141). Kidney transplantation too, had been performed earlier with no long term success (87) except in identical twins (114). With the realization that much can be done to prolong graft survival with immunosuppressive drugs (148) and with donor selection for a close genetic make-up to the recipient (36) this procedure became at first a justifiable experimental approach and, later, a world-wide accepted form of therapy for chronic renal failure.

Kidney transplantation is unique compared with allografting of other organs. In the first place, the kidney is a paired organ and this allows a living donor to give a kidney to a related member of his family without compromising his health or shortening his life span. Secondly, patients with chronic renal failure can now be kept alive on hemodialysis while waiting for a suitable kidney. It may be questioned why transplantation is done if the present mode of therapy with hemodialysis is so effective, and in fact, there are many centres in Canada and in the United States without transplantation facilities. Their patients are treated only by hemodialysis. The reverse is also true; for example the University of Minnesota

offers only a transplantation program with no maintenance hemodialysis, though they have hemodialysis facilities to prepare their patients for transplantation. Ideally, these two forms of therapy should be integrated for best results. Finally, patients who fail their transplant can still survive on dialysis treatment while waiting for a second, or a third transplant.

There is no question that hemodialysis can prolong the lives of chronic renal failure patients, some for as long as 10 - 15 years. There are, however, some aspects of the chronic renal failure problem which cannot be solved by hemodialysis, such as anemia, pruritus, osteodystrophy, insomnia, neuritis and impotence. These problems only get worse with the duration of dialysis treatment and can be cured only with a successful renal transplant. Hence, a successful renal transplant should be the ultimate effort for treating patients with chronic renal failure.

B. Factors influencing graft outcome.

According to the ninth report of the human renal transplant registry, renal transplant results reached a plateau in the year 1967 (124). In fact, Terasaki recently claimed that transplant survival rates are declining (129). Much of the earlier improvements in graft success had been attributed to improvements in immunosuppressive therapy, perfection in surgical technique and better patient management (99). The role of tissue typing has been questioned (11, 99, 116, 129) and the policy of blood transfusion has remained a controversy (57, 100, 127). The last two items will be further discussed below.

1. Histocompatibility.

The uniform excellent result of living-related-donor kidney transplants that are well-matched for the serologically defined antigens of the A & B loci of the HLA leaves no doubt that compatibility for the HLA is a major factor for graft success. Results in unrelated cadaver-donor kidney transplants are not conclusive as to whether or not compatibility at the A & B loci of the HLA is valuable as a predictor of graft survival because of contrasting findings of those in Europe and in North America. Thus, Oliver (126), van Hooff (179) and Dausset (37) and their associates obtained clear cut evidence of superiority of HLA (A & B) matching in graft outcome while Mickey (116) and Belzer (11) and their colleagues found no difference in outcome between matched and mismatched grafts. This

discrepancy has been explained on the basis of linkage disequilibrium between the A & B locus antigens and antigens of the HLA-D in Europe whereas such linkage might be rare in North America because of the genetically much more diverse population (180). This explanation also implies that it is not the HLA antigens of the A & B (and probably the C) loci that are important, but the MLC determinants or antigens of the D locus closely lined to the B. In support of this are the European findings that compatibility at the B locus alone resulted in good graft outcome (28, 126, 179). Skin grafting experiments are also in favour of this interpretation (26,188). Therefore, at the present, there is a general belief that matching for the MLC deteminants or D locus antigens of the HLA is more important for graft success than matchings for antigens of HLA-A and B.

2. The immune response of the recipient and the controversy over the effects of blood transfusion.

From the discussion just made on the role of HLA antigens, it is not surprising that transplants performed without a good match stand a good chance of being rejected. What is not yet explicable is the finding that many kidneys transplanted despite a total mismatch enjoy a prolonged survival with good function. This intriguing finding, together with the recent discovery in the mouse, of genetic control of immune response to synthetic polypeptides (110), led to the formulation of the concept of responders and non-responders in transplanted patients. This concept was put forward by Opelz and

his associates (128) and has gained some support (100). Based on the failure to detect cytotoxic antibodies against random cells, dialysis patients who subsequently received a kidney graft were classified into two groups according to whether or not they were transplanted before or after one year of dialysis treatment. Presumably all were sensitized to tissue antigens by blood transfusion and patients who could respond by producing cytotoxic antibody would do so within a year. Those who failed to produce cytotoxin within this period were identified as "non-responders". This group of patients had a transplant survival rate significantly higher than the other group.

There are several reasons why hemodialysis patients might be blood transfused. Firstly, they are all anemic; secondly, they may bleed from their cannula or fistula; thirdly, small amounts of blood are lost with each treatment; and finally, in the early days of hemodialysis, blood was used to prime a particular type of dialyser (coil hemodialyser) which held a large volume (500 ml). With improvement, this coil dialyser now holds much less (150 ml) and blood priming of the coil is no longer necessary.

There is no question that preformed cytotoxic antibodies induced by the incompatible white blood cells and platelets in multiple transfusions, in these hemodialysis patients, can be deleterious to the kidney graft, if the antibodies are directed to the graft (16, 93, 194).

Screening for cytotoxic antibody against a panel of random cells is thus of some predictive value of the difficulty likely to be encountered in finding a donor that is both HLA A & B compatible but also negative in the lymphocytotoxic cross-match test. Besides, patients may respond to blood transfusions by producing other non-complement fixing antibodies which may not be harmful and may even be beneficial. In this regard, Dossetor and his associates (45) have noted a paradoxical finding, in their 59 unrelated kidney transplants, that patients who were on dialysis longer and therefore received more blood transfusions prior to transplantation, were less likely to reject in the first three months. At that time (prior to 1967), screening for antibody was still not a common practise, so it was not clear whether these patients were capable of producing cytotoxic or other types of antibodies, or did they, in fact, become tolerant to tissue sensitization.

In this chapter, the author intends to present evidence that some of these so called non-responders who do not produce cytotoxic antibody do produce ABCIL antibody. Contrary to others' opinion that ABCIL antibody is harmful to renal allografts (4, 89, 170, 171), many of these patients with ABCIL antibody have been transplanted with good results. Specifically two had antibody directed against their donors, yet both kidneys have survived for more than three years with excellent function and no rejection episodes.

C. Materials and methods

1. Studies in hemodialysis patients

Sera from 22 patients with chronic renal failure from various causes were tested by 4 humoral tests indicated below, against a panel of the same 5 cells covering 15 of the known HLA-A & B locus antigens. The pertinent clinical data of these patients are presented in Table 17. In addition, serial studies were done on 11 patients using sera that had been stored at -70° C. By keeping this small panel of cells constant, it was possible to make meaningful analyses of comparative changes, serially in time, even without knowledge of the actual antibody specificities against HLA (A & B) antigens. All sera were inactivated by heating at 56° C for 30 min prior to testing in lymphocytotoxicity (CYT), capillary agglutination (CAT), antibody mediated cell dependent immun lympholysis (ABCIL) and microagglutination (MAGG).

The first three test have been described in the previous chapter. The last test was developed from the modified (J. Schlaut, unpublished data) leukoagglutination test of Payne (131) but carried out in microtitre trays like lymphocytotoxicity: 1 µl of leukocyte suspension (3 - 4 x 10^6 cells/ml) was mixed with 1 µl of test serum (under oil) for 2 min on a rotator. After 90 min of incubation at 37° C 1 µl of 6% acetic acid was added to lyse the contaminating erythrocytes. The mixture was then again subjected to the rotator treatment for 1 more min, allowed to stand for 5 min and read. The reaction was considered positive if

agglutination occurs.

2. Studies in transplanted patients.

To investigate the significance of ABCIL antibody in renal transplants, serial sera (4 - 12 in number, collected over a period of several months to several years) from 55 transplanted patients of known graft outcome were retrospectively tested against a constant panel of 4 - 5 cells in ABCIL. In addition, ABCIL cross-match was performed in 29 patients prior to transplantation concurrently with two other cross-matches: CYT and CML (cell-mediated lympholysis). This last test was done according to the method of Garovoy (70) using ^{51}Cr labelled peripheral blood lymphocyte targets. The attacking cells were mixed with the target cells at a ratio of 100 : 1 in 0.5 ml of medium 199 with 10% FCS, cultured for 4 hours at 37° C in 5% CO_2 atmosphere, and assayed in the same manner as described for ABCIL in Chapter II.

D. Results

1. Results from the comparison of 22 patients' sera at one point in time.

Of the 440 individual reactions performed with 22 sera by all four tests against five panel cells, 87 of the individual serum - cell reactions were positive (Table 17 and Fig. 13). Of these, positive reactions by CYT, CAT and MAGG were 21 (19.1%) 26 (23.6%) and 8 (7.3%) respectively. By combining these three tests, 27% of the individual serum-cell combinations showed positive reactions in one or more of the tests. The sera giving these positive reactions were from 11 of the 22 patients studied, indicating that 50% of the patients were sensitized by CYT, CAT or MAGG.

Also seen in Figure 13 is evidence that ABCIL alone was positive in 32 (29.1%) of the 110 serum-cell combinations, a figure slightly greater than the 27% presensitization reaction as detected by CYT, CAT and MAGG combined. When all 4 humoral tests are considered together, positive serum-cell combinations are 40/110 or 36%. The sera giving these positive reactions were from 13 of the 22 patients studied (59%). Thus, ABCIL allowed detection of presensitization in two additional patients of the 22.

2. Results of serial studies using stored sera from 11 patients.

Analyses of these data indicate that each individual patient may be in a different stage of response to tissue antigenic sensitization as a result of blood transfusion. By pooling the results of

Table 17. Pertinent clinical information and results of the 22 patients studied.

Patients	Age	Sex	Basic Disease[a]	Months on hemodialysis	Cumulative blood transfusion (unit)	Pregnancies	Prior transplant	Results[b] CYT	CAT	MAGG	ABCIL
BFIL	38	M	Analgesic nephopathy	9	0	-	-	-----	-----	-----	-----
RNES	52	M	CGN	10	1	-	-	-----	-----	-----	-----
RPUL	53	M	Polycystic kidney	50	16[c]	-	-	-----	-----	-----	-----
WREY	52	M	"	10	0	-	-	-----	-----	-----	-----
DSCH	16	F	CGN	4	2	-	-	-----	---+-	-----	-----
EDUC	33	F	CGN	1	6	8	-	-----	-----	-----	-----
LGRA	48	M	Nephrosclerosis	2	1	-	-	-----	-----	-----	-----
SSTE	17	F	CPN	2	0	-	-	-----	-----	-----	-----
JVAN	34	M	CGN	58	26[c]	-	-	-----	-+-+-	-+---	-----
BMIL	26	M	CGN	12	0	-	-	-----	-----	-----	-----
WMUI	48	M	CGN	12	3	-	-	-----	-----	-----	-----
RCLI	14	M	CGN	40	110	-	2	+++++	+++++	+++++	+++++
SEWA	28	M	CGN	37	84	-	-	--++-	---+-	-----	--++-
MKEI	52	M	CGN	17	29	-	-	-----	-----	-----	+---+
RSCH	48	F	Polycystic kidney	27	21	-	-	-++++	+++++	--+-	+++++
LCAR	60	M	CGN	17	20	-	-	+----	-----	-----	-----
WCHA	59	M	Hypertension	34	24	-	-	-----	-----	-----	---++
LNOR	29	F	CGN	37	162	-	2	--+--	+-+++	-----	-++++
DSWA	24	M	CGN	22	78	-	-	-+---	-+---	-----	++-++
JFAR	43	M	T.B. neph.	70	156	-	-	+-++-	+-++-	---+-	+-+++
HNIS	50	F	Analgesic neph.	4	1	1	-	-+---	+--+-	-----	--+-
AOLS	23	M	Familial neph.	46	126	-	-	+--++	+---+	+----	-+-++

[a] CGN = chronic glomerulonephritis
CPN = chronic pyelonephritis

[b] = same 5 cells in same order under each test

[c] = less than 5 transfusions in previous 18 months

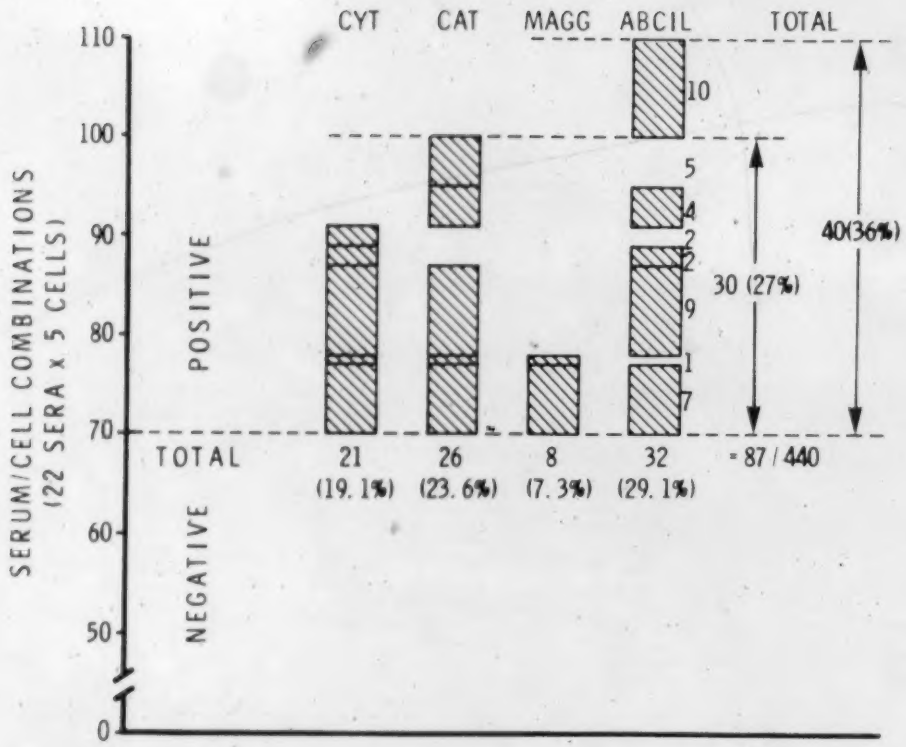
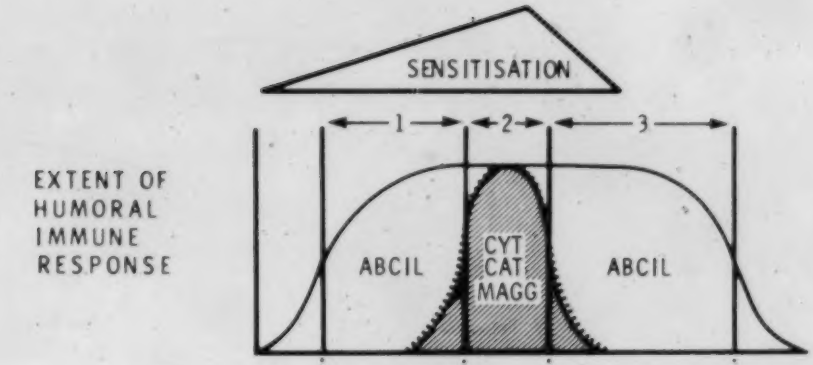


Figure 13. Comparison of CYT, CAT, MAGG, and ABCIL reactions of 22 hemodialysis patients' sera against five panel cells.

the 4 humoral tests and correlating them with the clinical course, it is possible to construct a diagram which depicts the humoral immune response of these patients to sensitization (Fig 14). Thus, patients FANO and VGRU fit in the early phase of immune response to sensitization (stage 1 in the diagram); patients KBAB, RCLI, and JFAR fit in the peak phase (stage 2); and patients DHAG, PWIL, and HHEY fit in the late or decrudescent phase (stage 3). Patients SEWA fits in both early and peak phases, patients GMOS fits in both peak and descrudescent phases, and APAR fits in all three phases. In such schematization individual patients vary greatly as to the length of time they were in a given phase or the extent of humoral immunity expressed against the panels. Patients SEWA, KBAB, DHAG, PWIL and APAR will be further described below to give more details concerning these phases of humoral immune responsiveness.

a) Patient SEWA (Table 18), a 40 year old male with chronic glomerulonephritis, was first tested after 4 months on hemodialysis by which time he had been transfused with five units of blood. At this time ABCIL was positive with 2 of the 5 cells tested. CYT was not positive until the third screen at 21 months on dialysis and after 50 units of blood. The CYT reactions then persisted to the last screen at 38 months. At this time ABCIL had disappeared. Blood transfusions ceased to become necessary after 32 months. These various serial reactions would therefore represent the immune response of this patient to blood transfusion from the early to the peak phase.



AGE	SEX	DIAGNOSIS	period (months) between 1st & last test	BLOOD GIVEN (UNITS)
25	M	CGN	0 ← FANO → 12	70
14	M	OBST. NEPH.	-4 ← VGRU → 22	48
38	M	CGN	4 ← SEWA → 38	80 - TABLE 3
39	M	CGN	47 ← KBAB → 77	90 - TABLE 4
14	M	CGN	10 ← RCLI → 41	110
45	M	T.B. NEPH.	46 ← JFAR → 76	156
46	F	CGN	5 ← GMOS → 35	30
30	M	CGN	32 ← DHAG → 73	230 - TABLE 5a
41	M	CGN	33 ← PWIL → 68	90 - TABLE 5b
44	M	CGN	24 ← HHEY → 52	110
38	F	CGN	-3 ← APAR → 27	95 - TABLE 6

Figure 14. Diagram depicting the humoral immune response to tissue sensitisation by blood transfusion of 11 hemodialysis patients who were studied serially. Arrows between dotted lines indicate hypothetical phase(s) during which they were studied with periods (months) between the first and the last tests indicated at each end. Additional details on five of these patients are given in Tables 18 - 22.

Table 18. Patient SEWA, a 40 yr old male with chronic glomerulonephritis.

Tests	Reactions[a]						
	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE	ABCDE
CYT	-----	-----	--+++	--+++	--+-+	--+++	--+++
CAT	-----	-----	--+-+	--+-+	--+--	--+--	--++-
AGG	-----	-----	----+	----+	-----	-----	----+
ABCIL	---++	---++	----+	---++	----+	----+	-----
Months on Hemodialysis	4	12	21	26	32	35	38
Cumulative Blood Transfusions	5	20	50	70	80	80	80
Clinical Events							

[a] Against a constant five cell panel, arranged in the same order at each period tested; HLA(A&B) profiles as follows:

Cell A = A11,AW32,B12,BW35

Cell B = A1,A11,B7,B8

Cell C = A28,AW32,B14,BW22

Cell D = A10,AW32,BW16,BW17

Cell E = A2,A9,B13

b) Patient KBAB (Table 19), a 41 year old male with chronic glomerulonephritis, had positive reactions by all 4 tests to almost all of the panel cells when first tested after 47 months on dialysis, by which time he had already received 80 units of blood transfusion. These widespread reactions remained unchanged when tested 30 months later, despite the fact that no additional transfusions were given during the 20 months preceding the last screening test.

c) Patient GHAG (Table 20), a 32 year old male who after 32 months on dialysis and 150 units of blood transfusion had one positive CYT, two positive CAT, and one positive MAGG reaction against the panel whereas ABCIL was positive against 4 of 5 cells. After 80 more blood transfusions and after splenectomy (2 months later), no more blood transfusions were required. CYT, CAT and MAGG became undetectable at this point, but ABCIL reactions were still positive 20 months after the last transfusion.

d) Patient PWIL (Table 21), a 43 year old male with chronic glomerulonephritis. His pattern has many similarities to DHAG in that he received many blood transfusions in the 33 months prior to first being studied and, at that time, had CYT antibodies as well as other evidence of sensitization. Then CYT antibodies became undetectable by the panel despite continued transfusion. His pattern differs from DHAG in the widespread and persistent evidence of CAT antibody, although both showed ABCIL antibodies that at some stage mark all five panel cells. ABCIL antibodies persisted longer than CAT, being still positive against 2 of 5 panel cells at 21 months

Table 19. Patient KBAK, a 41 yr old male with chronic glomerulonephritis.

Tests	Reactions[a] CDEBA	CDEBA	CDEBA	CDEBA	CDEBA
CYT	-++++	-++++	--+++	--+++	--+++
CAT	+++++	--+++	-++++	--+++	+-+++
MAGG	--+++	--+++	--+++	--+++	--+++
ABCIL	-++++	-++++	-++++	-++++	-++++
Months on Hemodialysis	47	53	58	68	77
Cumulative Blood Transfusions	80	85	90	90	90
Clinical Events			* Parathyroidectomy		

[a] See footnote [a], Table 18.

Table 20. Patient DHAG, a 32 yr old male with chronic glomerulonephritis.

Tests	Reactions[a] CADBE	CADBE	CADBE	CADBE	CADBE	CADBE	CADBE	CADBE
CYT	----+	----+	----+	-----	-----	-----	-----	-----
CAT	---++	--+-+	-----	-----	-----	-----	-----	-----
MAGG	----+	-----	-----	-----	-----	-----	-----	-----
ABCIL	-++++	-++++	-++++	+++++	+++++	+++++	+++++	-++++
Months on Hemodialysis of after Transplantation	32	45	50	53	59	69	72	- 1
Cumulative Blood Transfusions	150	190	220	230	230	230	230	230
Clinical Events				* Splenectomy			* Renal Transplantation	

[a] See footnote [a], Table 18

Table 21. Patient PWIL, a 43 yr old patient with chronic glomerulonephritis.

Tests	Reactions[a]									
	BEACD	BEACD	BEACD	BEACD	BEACD	BEACD	BEACD	BEACD	BEACD	BEACD
CYT	---++	---++	-----	-----	-----	-----	-----	-----	-----	-----
CAT	+++++	++-++	+++++	+++-+	+++-+	+++-+	--+--	--+--	+-+--	-----
MAGG	----+	--+-+	-----	-----	-----	-----	-----	-----	-----	-----
ABCIL	+++++	+++++	++-++	+++++	++-++	++-++	++-++	+++++	+++++	---++
Months on Hemodialysis or after Transplantation	33	35	44	46	47	51	53	59	60 65	- 3
Cumulative Blood Transfusions	55	55	80	85	90	90	90	90	90	90
Clinical Events			* Septicemia		* Splenectomy				*Renal Transplantation	

[a] See footnote [a], Table 18.

after splenectomy had abolished the need for transfusions.

e) Patient APAR (Table 22), a 40 year old female with chronic glomerulonephritis whose sera showed widespread positive ABCIL reactions after 6 months on dialysis during which she had received 30 units of blood. CYT, CAT, and MAGG performed during this period of time were uniformly negative. Only in the next screen 5 months later (after 11 months on dialysis) did the latter become detectable against 2 of the 5 cells. Subsequently the 4 humoral systems were inconsistently positive up to the time of transplantation. When tested 5 months after transplantation only one ABCIL reaction was positive. The author believes these serial reactions represent all 3 phases of immune response of this patient, although there is no identifiable peak by CYT.

3. Results in renal transplants

a) ABCIL reactivity of transplanted patients against random cells. Analyses of these data showed that 26 patients failed to give any positive ABCIL reactions when tested over a period of 4 - 58 months and 10 of these patients rejected their graft within one year. Five patients give 1 - 2 positive reactions over 7 - 33 months and one kidney was rejected at two months. The rest of the patients (24) had 3 or more positive ABCIL reactions over 1 - 59 months and 7 kidneys were rejected within a year. Thus, there was no difference between patients with ABCIL antibodies and those without, and their graft outcome.

Table 22. Patient APAR, a 40 yr old female with chronic glomerulonephritis.

Tests	Reactions[a]								
	CAEDB	CAEDB	CAEDB	CAEDB	CAEDB	CAEDB	CAEDB	CAEDB	CAEDB
CYT	-----	-----	+---+	----+	-----	---++	----+	----+	-----
CAT	-----	-----	+---+	----+	+---+	+---+	----+	--+++	-----
MAGG	-----	-----	----+	----+	----+	----+	----+	----+	-----
ABCIL	--++-	+-+++	--+++	--+++	--+++	-++++	-+-++	---++	----+
Months on Hemodialysis or after Transplantation	-3[b]	6	11	12	15	19	22	22	- 5
Cumulative Blood Transfusions	10[c]	30	50	50	60	80	85	85	85
Clinical Events								* Renal Transplantation	

[a] See footnote [a], Table 18.

[b] Three months before onset of hemodialysis.

[c] Blood given over previous 3 years.

b) ABCIL cross-match. Table 23 shows the various immunologic parameters including specific reactions against kidney donor in 29 transplanted patients and their graft outcome. CML cross-matches were uniformly negative. Ten kidneys were rejected, two in an accelerated fashion and, retrospectively, both recipients (BRAW and CROB) had a positive CYT cross-match. In 6 patients with a negative CYT cross-match but positive ABCIL against random cells, only one (EGLI) rejected his renal graft. Among 7 patients with a negative ABCIL status (against random cells) who rejected their transplants, 3 (BSHA, ESMI and BMIL) turned ABCIL positive but all 3 also developed CYT against random cells. The ABCIL status of the other 4 (DSME, WKUZ, DFER and JPAT) remained negative. Finally, two patients (HNIS and PWIL) had a positive ABCIL but negative CYT cross-match and both patients' transplanted kidneys have survived for more than 3 years at the time of this writing.

Table 23. ABCIL antibody status, CYT, ABCIL and CML cross-matches and graft outcome in 29 renal transplants.

Patients	ABCIL vs random cells	Cross-matches CYT	ABCIL	CML	Graft & Function outcome[a]
FAIN	-	-	-	-	Surviving, 4 yrs; Cr = 1.0
SBEG	-	-	-	-	Surviving, 29 mo; Cr = 1.0
WCHA	-	-	-	-	Surviving, 27 mo; Cr = 1.0
IDRI	-	-	-	-	Surviving, 23 mo; Cr = 1.1
EDUC	-	-	-	-	Surviving, 32 mo; Cr = 1.0
EGLI	+	-	-	-	Rejected at 8 mo
EHIN	-	-	-	-	Surviving, 25 mo; Cr = 0.6
RKRA	-	-	-	-	Surviving, 4 yrs; Cr = 2.0
ANAN	-	-	-	-	Surviving, 18 mo; Cr = 0.8
APAR	+	-	-	-	Surviving, 41 mo; Cr = 2.8
ZSAI	-	-	-	-	Surviving, 42 mo; Cr = 1.7
DSCH	+	-	-	-	Surviving, 28 mo; Cr = 2.5
GSCH	-	-	-	-	Surviving, 28 mo; Cr = 2.9
JSIN	-	-	-	-	Surviving, 23 mo; Cr = 0.9
SSTE	-	-	-	-	Surviving, 29 mo; Cr = 1.1
OREY	-	-	-	-	Deceased, not rejected
BSHA	+	-	-	-	Rejected at 2 wk
ESMI	-	-	-	-	Rejected at 5 wk
DSME	-	-	-	-	Rejected at 6 wk
WKUZ	-	-	-	-	Rejected at 7 wk
VGRU	+	-	-	-	Surviving, 12 mo; Cr = 0.7
BMIL	-	-	-	-	Rejected at 3 mo
WMOG	-	-	-	-	Surviving, 13 mo; Cr = 1.4
DFER	-	-	-	-	Rejected at 1 wk
JPAT	-	-	-	-	Rejected at 3 mo
HNIS	+	-	+	-	Surviving, 3 yrs; Cr = 1.0
PWIL	+	-	+	-	Surviving, 38 mo; Cr = 2.0
BRAW	+	+	+	-	Rejected, hyperacutely
CROB	+	+	+	-	Rejected, hyperacutely

[a] As measured by serum creatinine level.

E. Discussion

The detection of tissue sensitization by CYT in the one-shot study (in 9/22, or 41% of the patients) is higher than in other series (164) even though only a small panel of 5 cells was used and may be a reflection of the higher requirement of blood transfusions in these patients due to shortened red cell survival caused by a constituent of untreated Edmonton water, probably chloramine (175) (prior to deionization of all water used in hemodialysis in September 1972). However, CYT by itself is inadequate to detect all humoral evidence of tissue sensitization. With the additon of CAT and ABCIL, detectable sensitzation becomes 11/22 or 50% and 13/22 or 59%, respectively. Thus, with ABCIL and a five-cell panel, sensitization was detected in 2 additional patients. These 2 patients were among those 10 who had been on dialysis for more than 1 year and had received more than five units of blood transfusion. In the other 8 patients in this category, evidence of sensitization was detected by CYT alone. Thus, with the addition of the ABCIL system, sensitization was detectable in all 10 patients who had received more than 5 units of blood transfusion in the previous year.

This modest increase in the number of patients found to have sensitization is not the main reason for advocating ABCIL, as these two sera might well have given evidence of existent tissue sensitization by using a larger CYT test panel. The value of the additional test is at once apparent if one considers the individual ABCIL-

positive CYT-negative reactions as being a potential or theoretical pretransplant cross-match reaction.

From the serial study it is clear that different individuals may have different patterns of humoral immune response to tissue sensitization by blood transfusion. Only 4 of 11 patients (KBAB, RCLI, JFAR, and GMOS) appear to respond by producing widespread antibodies including CYT to more than half of the cells tested over a prolonged period. On the other hand, in 4 patients who were tested in their early course on hemodialysis, ABCIL appeared earlier than CYT. In certain individuals (such as SEWA), ABCIL may disappear before CYT. But in general the reverse is true. Thus, in all 5 patients tested late in their course, after tissue sensitization by blood transfusion could be stopped, ABCIL persisted longer. Hence, CYT reactions tend to appear later with sensitization and disappear earlier after cessation of immunization than ABCIL reactivity. It is also clear that patient DHAG, if screened for CYT from the 53rd to the 72nd month, would be labelled as a "non-responder", although we know retrospectively that he had positive CYT reactions earlier against one panel cell; however, in contrast, ABCIL reactions were widespread and persistent. This can be explained in part by the extreme sensitivity of this test, as well as the wider cell spectrum of reactivity as shown in Chapter III.

The earlier studies (Chapter III) with ABCIL antibodies in the sera of parous women showed that they persist longer than CYT antibody

after cessation of antigenic stimulation. The present study has not only confirmed this but has also suggested the earlier appearance of this antibody after antigenic exposure. This earlier appearance and longer persistence, in addition to the wider spectrum of panel cell reactivity, make this system an important adjunct to tests of lymphocytotoxicity and leukoagglutination for detecting sensitization in potential transplant recipients.

The success or failure of any organ graft is a complex problem in which HLA matching plays a role but is far from providing the entire answer. Intrinsic immune responsiveness may govern the ability of a recipient to mount a rejection on the graft (183). The concept of nonresponders (128), as judged by the absence of CYT in the sera of patients on dialysis for more than one year with remarkable correlation with graft success thus sounds attractive. However, many of the so called "nonresponders" are, in fact, "different responders" in that they do not produce CYT but do produce other types of antibodies. Perhaps some of these non-cytotoxic antibodies may be protective to the graft; this may explain why some patients with more dialysis do better after transplantation (45).

In contrast to others' claims that LDA (ABCIL) is bad in kidney transplantation (4, 89, 170, 171), the author finds no conclusive evidence from this study either by judging from the positive ABCIL reactions against random cells or by direct ABCIL cross-matching. Experiences of the group in Boston (23) and in Paris (42) are in agreement with this. Although LDA (or ABCIL)

had been postulated of being responsible for an irreversible rejection in one patient (159), the same group of investigators now have evidence to suggest that this antibody is more associated with quiescence than with rejection episodes (158). Such discrepancy in results of one group of investigators from another may be due to technical differences, such as macro-technique vs micro-technique, PBL target vs PHA blast target, good effectors vs not-so-good effectors, etc. But in view of the findings in the previous chapter that some ABCIL antibody may have specificities against A and B antigens of the HLA while others may be entirely directed against D locus antigens, it is tempting to speculate that in renal transplants there may be two types of ABCIL antibody, those with HLA-A & B specificities may be harmful and those with non-A & B specificities may not be; or, more specifically, this latter category may even be beneficial, if directed against D locus antigens or MLC determinants.

In this regard, patient PWIL's ABCIL activity against his donor could not be removed with donor platelets though it is absorbable with donor lymphocytes, thus suggesting the non-HLA-A & B nature of the antibody. He received the kidney from his 60 year old father and never had any rejection crises since transplantation with a serum creatinine of 2.0 mg% from the start and his serum creatinine has remained at about 2.0 mg%. Because of the many blood transfusions he received during dialysis, this patient represents a hyperimmunized

individual who is doing well after transplantation. The reason his transplant is doing so well is obscure and the fact that he has ABCIL antibody against the donor may be quite irrelevant. He may, for example, have suppressor cells actively participating in a process which protects the graft. The antibody may be directed against a membrane determinant on lymphocytes which is not a transplantation antigen. On the other hand, it may be directed against HLA-D. Studies are being done by using this serum in family segregation, and by reacting this serum against HLA-D homozygous cells to investigate this possibility.

Patient HNIS received a cadaver-donor kidney. Likewise, she is doing extremely well without experiencing any rejection episodes in the presence of a positive ABCIL cross-match. Prior to transplantation she received only 1 unit of blood though she had borne 3 children. Unfortunately, donor platelets were not available for absorption study. The only mismatch is antigen B-12 but, by reacting this serum with unrelated cells with and without B-12, only one in three with antigen B-12 was positive. Neither the platelets nor lymphocytes from this B-12 ABCIL positive individual could remove the antibody activity against the donor. Therefore, in this case too, the ABCIL antibody is not directed against A and B antigens of the HLA.

It is hoped that through a cooperative effort among kidney transplantation centres, a definite role of ABCIL antibody of both specificities, HLA-A & B and HLA-D, can be established. Recipient sera

from HLA (A & B) haploidentical sibling and parent child transplantations can be retrospectively studied with platelet absorption provided that donor platelets and lymphocytes, and pretransplant recipient sera can be obtained. Such an effort has been planned.

F. Conclusion

Chronic renal failure patients maintained on dialysis are exposed to tissue sensitization by blood transfusion and can produce different kinds of antibody.

A comparison of these different antibodies produced as studied serially in these patients indicates that ABCIL antibody appears earlier and disappears later than CYT. The biologic significance of ABCIL in transplantation has yet to be determined; there is no definite evidence to indicate that it is deleterious to renal allografts, nor has its enhancing role been clearly established.

Chapter V. General Discussions and Conclusion

A. General Discussions

The finding that a lymphoid subpopulation can cooperate with an antibody to mediate target cell destruction is of considerable interest because it strongly suggests that certain chronic pathological conditions with round cell infiltration of unknown cause may be the result of this type of immunological injury. In fact, one typical example is Hashimoto thyroiditis and recently, Fakhri and Hobbs were able to show, using normal thyroid tissue pretreated with serum from such patients, the attachment of lymphoid cells to the thyroid tissue. Thus, this immune system was implicated as having a pathogenetic role in this disease (53). It is not known whether other chronic inflammatory conditions of obscure etiology, such as chronic pyelonephritis, may also involve this type of autoallergic phenomenon. This issue is significant not only for better understanding of a disease process but also from the therapeutic standpoint.

The extreme degree of sensitivity of this immune system would further suggest its biologic significance such as, in dealing with foreign invasions by micro-organisms or in defense against tumor in a highly economical fashion. The antibody has been repeatedly shown to belong to an IgG class (60, 108, 109); IgM antibodies are not effective. It appears that many cell types of the non-T subpopulation can function as an effector in mediating cytotoxicity

and the studies in Chapter II indicate further that not all effector cells are capable of destroying target of a particular type. The reason for this differential selective killing capacity is not understood. It may simply reflect the degree of differentiation of this type of cells in the evolutionary process. Thus, while human K cells (K1, non-adherent) are active against a wide variety of targets, including lymphocytes sensitized with anti-HLA antibodies, chicken but not human erythrocytes, many cell lines and some tumor cells, mouse K cells (K2, adherent) are only active against chicken red blood cells (106). In this regard, it is interesting to note that while this immune system is highly sensitive in detecting allosensitization in human, the author has consistently failed to observe this type of immunity in similar situations in mice. This conclusion has also been reached by other investigators (77).

It is becoming apparent that other non-lymphoid cells are also capable of mediating antibody dependent lysis, such as granulocytes and certain non-lymphoid cell lines (106). It is not certain whether blood platelets, by virtue of their Fc receptors, can also carry out this lytic function. The author had done some experiments in this regard but the results were not conclusive.

B. Clinical applications

1. ABCIL as a transplant cross-match procedure.

This has been discussed in detail in the previous chapter. In view of the finding in Chapter III that there are two types of ABCIL antibodies following tissue sensitization, one with specificities directed against HLA-A & B (& C) antigens and the other, against HLA-D; and since the biologic significance of these two types of ABCIL antibody is not yet clearly established, it seems logical to explore this further by carrying out the cross-matching with and without donor platelet absorption of the recipient sera, in conjunction with other cross-matches.

2. The use of the ABCIL technique to study disease association with HLA.

One bonus of HLA research is the finding that many diseases are associated with this major histocompatibility system, some very strongly, especially those of rather obscure etiology such as ankylosing spondylitis, psoriasis, multiple sclerosis and diabetes mellitus. Most of these diseases were studied using the lymphocytotoxicity test and the antigens reported are those of the A and B loci. But it is not the A and B locus antigens that are implicated as being associated with these diseases. Rather, the loci for immune response (Ir) genes, mapped in this chromosomal region and in closer proximity with the B and D loci than the A, are more likely candidates for this association (160). The apparent association of these diseases with certain A and B antigens may be due

to linkage disequilibrium of the A and B alleles with the Ir gene (these genes are also called disease predisposing genes). This would also explain why most disease associated antigens belong to the B locus (though a few are associated with the A locus suggesting that there may be Ir genes in close proximity to locus A as well), and the evidence is strong that this disease association is even greater with D locus antigens as detected by the MLC test using HLA-D homozygous cells. This is true for multiple sclerosis which has a stronger association with HLA-DW2 (previously LD-7a) than with HLA-B7.

In view of the finding that ABCIL may detect HLA-D antigens (Chapter III), ABCIL sera of such specificity may be able to mark individuals with certain HLA associated disease. This may be helpful in making a diagnosis or in prognostication. Since Ir gene products may be expressed on lymphocytes, it may also be possible to detect these I region associated (Ia) antigens directly; and the source to look for such antisera would be in multiparous wives of husbands afflicted with these diseases.

3. Study of the effector cell activity (ECA) in various disease states and the effect of drugs.

Using two different antibody target systems as described in Chapter II, it was found that chronic renal failure patients have low ECA against an antibody coated lymphocyte target though their ECA against antibody sensitized red blood cells was quite normal.

It thus appears that measuring ECA with the former system may be a sensitive indication for immunosuppression, presumably due to uremia in this case. Similar results had been obtained in immunosuppressed renal transplants, many of them had zero activity when they were doing well. Although, there was suggestive evidence that ECA (using a lymphocyte target system) might rise in some renal transplants at the time of rejection crisis, this finding had not been consistent and might be reflecting their immune status rather than being responsible for rejection. That ECA may reflect immunosuppression is further supported by the observation in the following two patients, one of them received high dose intravenous methylprednisolone for rejection and serial studies documented a progressive fall in ECA with treatment. The other patient had acute dermatomyositis with enhanced ECA, after receiving 1 gram cyclophosphamide intravenously, ECA fell to almost zero.

It is conceivable that in certain immunodeficiency states this Fc receptor bearing cell population may be lacking selectively or in association with deficiency of other cells; thus far this has not been the case. In a few hypogammaglobulinemic children studied, their ECA, though low, were normal for age. In contrast seven patients with myelofibrosis had zero to near zero ECA. It is not yet possible to relate this finding to myeloproliferative disorders as a group, but it suggests that measuring ECA in this immune system may add another facet to the study of patients with disorders of

various organ-systems and this may lead to better understanding of some disease processes as well as to better diagnosis and management, particularly in the field of neoplasia and autoimmunity. It would also be worthwhile to study effects of drugs to ECA, especially cancer therapeutic agents.

4. The use of the ABCIL system in the detection of immune complexes.

The predominant cause of terminal renal failure requiring dialysis and transplantation is glomerulonephritis in its many forms, most of which have been clearly shown to have an immunologic basis. Dixon (44) has stressed two principle mechanisms of immunologic injury. They usually cannot be differentiated from each other on clinical grounds. The first results from antiglomerular basement membrane (GBM) antibody reacting with GBM and activating the complement system to produce renal damage --- Masugi type glomerulonephritis. Only 5 - 15% of clinical glomerulonephritis belongs to this type. The second mechanism responsible for the majority of cases of glomerulonephritis results from deposition of antigen-antibody complexes in the glomeruli with secondary injury to the kidney. This type includes those associated with known antigens: acute post-streptococcal glomerulonephritis, lupus nephritis, nephritis resulting from serum sickness or from persistent baterial infection such as subacute bacterial endocarditis, etc. Also included in this category are forms of glomerulonephritis in

which the antigens in the complex are quite unknown: membrano-proliferative glomerulonephritis (non-streptoccal), membraneous glomerulonephritis, rapidly progressive glomerulonephritis, Berger's IgA - IgM focal nephritis.

Based on the blocking capability by antigen-antibody complexes on the effector cell function in this immune system, Jewell and MacLennan (91) has reported the use of this system in the detection of immune complexes in the sera of patients with chronic inflammatory bowel disease. The immune complexes so detected were of small size in contrast to other methods which detect larger sized complexes (92, 123, 134, 165). In this regard, blocking of ECA in this system might be useful in detecting immune complexes of small size which might have been missed using other methods. Immune complexes of small size might be filtered and excreted in the urine though, to-date, evidence in support or against this is lacking; it may be worthwhile to look for complexes in urine of patients with various forms of glomerulonephritis using this technique.

C. Conclusions

The following conclusions can be drawn from the studies in this thesis: -

1. There are two types of effector cells in this antibody mediated cell dependent lytic system: 1. lymphocytic; these are radiosensitive, do not adhere to glass or plastic, and are active to lymphocyte targets coated with alloantibodies. 2. Monocytic; these are radioresistant, adherent to glass or plastic, and are active to erythrocyte targets sensitized with anti-AB antibodies.

2. There is an age and sex difference of lymphocytic effector cells; there is no sex difference amongst monocytic effector cells. The former, but not the latter, are also sensitive to immunosuppression.

3. Compared with the lymphocytotoxicity (CYT), ABCIL is a more sensitive method of detecting allosensitization in human. Following sensitization, ABCIL antibody appears before CYT antibody; after cessation of antigenic exposure, ABCIL antibody also persists longer.

4. Different individuals may respond to allosensitization by producing different types of antibody; some of the so-called "nonresponders" that do not produce CYT antibody are, in fact, "ABCIL responders" because they do produce ABCIL antibody.

5. There are two types of ABCIL antibody with regard to HLA specificity; those with HLA-A & B (and probably C) specificities are directed to both T and B cells in peripheral blood. Those with presumably HLA-D specificities are directed only

to B cells, the stimulating cells in MLC. In this regard ABCIL represents a rapid serological technique for detecting HLA-D antigens

6. The biologic significance of these two types of ABCIL antibody in the field of kidney transplantation is, as yet, not clear.

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Appendix A. Major Histocompatibility Complexes of Mammalian Species

Species	Designation	Estimated Knowledge, 1974*
Mouse	H-2	++++
Rat	H-1 or Ag-B	++
Guinea pig	GPL-A	(+)
Rabbit	RL-A	(+)
Pig	SL-A (PL-A)	+
Sheep	ShL-A	(+)
Dog	DL-A	+++
Rhesus monkey	RhL-A	+++
Chimpanzee	ChL-A	++
Man	HL-A	+++

* Degree of knowledge regarding SD. LD, and Ir lcci of the complex.

From: H. Balner. Choice of animal species for modern transplantation research. Transplant. Proc. 6, 19, 1974.

Appendix B. Genetic Nomenclature of the H-2 Complex*

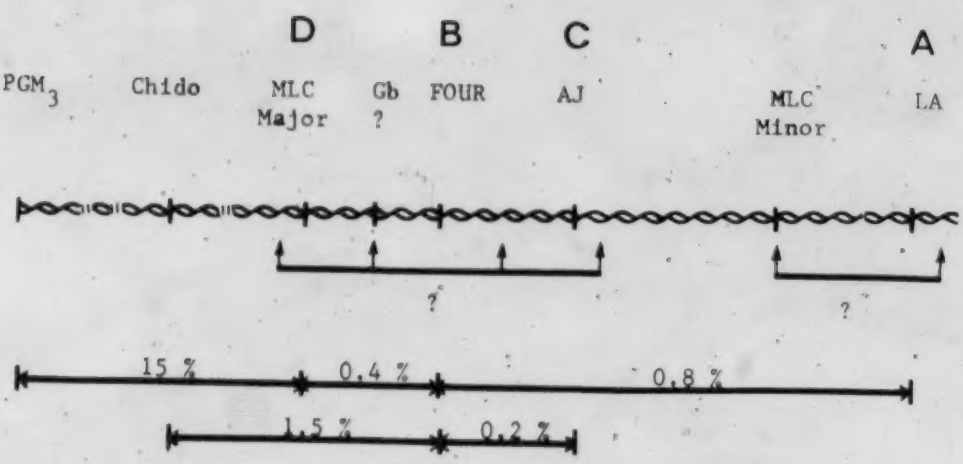
Complex	H-2						
Ends	K						D
Regions	K		I		S	"X"	D
Subregions		IA	IB	IC			
Loci	H-2K	Ir-1A	Ir-1B	Ia	Ss	Lad	H-2D
	Lad	Ir(T,G)A-L**	Ir-LDH$_B$	Lad	Slp	H-2G	Lad
		Ir-IgA**	Ir-Nase	Ir-GLT?			
		Ir-RE**	Lad?				
		Ir-OA	Ia				
		Ir-OM					
		Ir-BGG					
		H-2I					
		Lad					
		Ia					

* The genetic relationship of the individual loci in each region or subregion is not known.

** The location of this gene into the IA subregion is only tentative.

From: J. Klein. Biology of the mouse histocompatibility-2 complex. Springer-Verlag, N.Y., 1975.

Appendix C: Human MHC* (Chromosome no. 6)



* Showing approximate recombination frequencies.

Modified from: Teisberg, P., Olaisen, B., Gedde-Dahl, T., Jr. and Thorsby, E. On the localization of the Gb locus within the MHS region of chromosome no.6. Tissue Antigens 5, 257, 1975.

Appendix D. Complete Listing of Recognized HLA Specificities[a]

New	Previous[b]	New	Previous	New	Previous	New	Previous
HLA-A1	HL-A1	HLA-B5	HL-A5	HLA-Cw1	T1	HLA-Dw1	LD 101
HLA-A2	HL-A2	HLA-B7	HL-A7	HLA-Cw2	T2	HLA-Dw2	LD 102
HLA-A3	HL-A3	HLA-B8	HL-A8	HLA-Cw3	T3	HLA-Dw3	LD 103
HLA-A9	HL-A9	HLA-B12	HL-A12	HLA-Cw4	T4	HLA-Dw4	LD 104
HLA-A10	HL-A10	HLA-B13	HL-A13	HLA-Cw5	T5	HLA-Dw5	LD 105
HLA-A11	HL-A11	HLA-B14	W14			HLA-Dw6	LD 106
HLA-A28	W28	HLA-B18	W18				
HLA-A29	W29	HLA-B27	W27				
HLA-Aw19	Li[c]	HLA-Bw15	W15				
HLA-Aw23	W23	HLA-Bw16	W16				
HLA-Aw24	W24	HLA-Bw17	W17				
HLA-Aw25	W25	HLA-Bw21	W21				
HLA-Aw26	W26	HLA-Bw22	W22				
HLA-Aw30	W30	HLA-Bw35	W5				
HLA-Aw31	W31	HLA-Bw37	Ty				
HLA-Aw32	W32	HLA-Bw38	W16.1				
HLA-Aw33	W19.6	HLA-Bw39	W16.2				
HLA-Aw34	Malay 2	HLA-Bw40	W10				
HLA-Aw36	Mo*	HLA-Bw41	Sabe11				
HLA-Aw43	BK	HLA-Bw42	MWA				

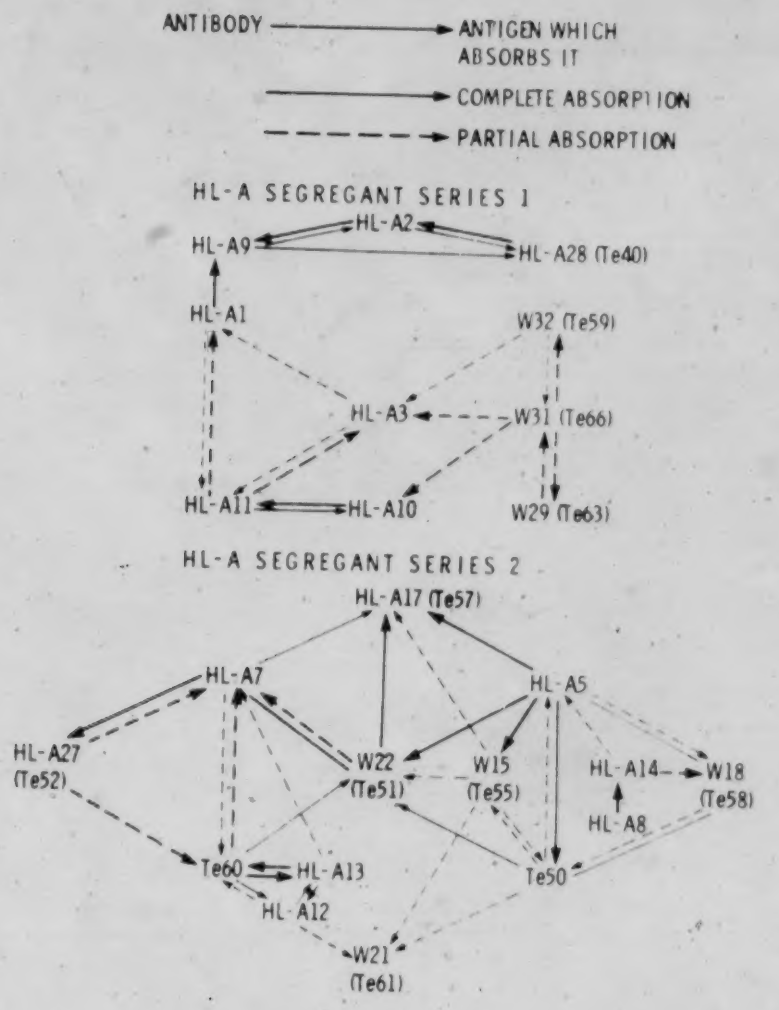
a) The previously reserved specificities W4 (4a) and W6 (4b) remain w4 and w6. These specificities are closely associated with the B locus

b) For more comprehensive listing of equivalents see table 1 and 'table of equivalent nomenclature' in Histocompatibility Testing 1972, p. 7.

c) HLA-Aw19 includes at least HLA-A29, Aw30, Aw31, Aw32, Aw33 and Aw34(?)

From: Histocompatibility Testing 1975, F. Kissmeyer-Nielsen, ed., Munksgaard, Copenhagen, 1975, p. 8.

Appendix E. Cross reactivity in the HLA-A and -B series.



From: Mittal, K.K. and Terasaki, P.I. Cross reactivity in the HL-A system. Tissue Antigens 2, 94, 1972, and personal communication.